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Sex Differences in HIV-1 Pathogenesis: Role of Steroid Hormones and Type I Interferon in $CD4^+$ T Cell Activation and Viral Control

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By

Elina El-Badry
B.S., The College of William & Mary, 2014

Advisor: Eric Hunter, Ph.D.

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Abstract

Sex differences play a role in the pathogenesis of a number of viral diseases. To date, the preponderance of data on the influence of sex in HIV-1 infection has been limited to the chronic phase of disease, and little is known about how sex differences influence acute HIV-1 infection. We have observed profound differences in viral load (VL) and $CD4^+$ T cell activation from the earliest time-points in men and women in a Zambian heterosexual acute infection cohort. Women exhibited a more than two-fold higher rate of $CD4^+$ T cell loss despite significantly lower VL than men. The importance of studying acute infection was highlighted by the observation that very early in infection, women exhibited significantly higher levels of $CD4^+$ T cell activation, a difference that was lost over the first 3 years of infection as activation in men increased. In women, activation of $CD4^+$ T cells in the acute phase was significantly correlated to plasma levels of 17β-estradiol (E2). E2 level in early infection was also associated with lower early and set-point VL in women, and was able to significantly restrict HIV-1 replication *in vitro*. In order to better understand the mechanism behind these observations, we compared gene expression between $CD4^+$ T cells from acutely HIV-1 infected men and women. We have identified a set of genes that are both more highly expressed in women and negatively correlated with VL, indicating that they may play a role in the comparative control of VL observed in women. This gene set is significantly enriched for type I interferon (IFN) signaling genes including *IRF7*, *DDX58*, *SAMHD1*, *OAS2*, and *TRIM14*.

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Chapter I: Introduction

Current Status of the HIV/AIDS Epidemic

In 1981, the Centers for Disease Control and Prevention (CDC) reported in its Morbidity and Mortality Weekly Report on several instances of young, homosexual men in Los Angeles who had contracted *Pneumocystis carinii,* a rare opportunistic bacterial infection. This was followed shortly thereafter by additional reports of *P. carnii* and other rare malignancies, including Kaposi's sarcoma, in additional men in in major cities throughout the United States. The affected individuals were found to be severely immunocompromised, leading to the subsequent designation of their condition as Acquired Immune Deficiency Syndrome (AIDS). In 1983, a novel retrovirus, later termed Human Immunodeficiency Virus Type I (HIV-1) was identified as the causative agent of AIDS. In 1984, United States Health and Human Services Secretary Margaret Heckler famously declared that a vaccine against HIV-1 would be available in two years. Her prediction never came true, and instead HIV-1 has proved to be one of the most devastating infectious diseases to emerge in recent history (1). Since its identification, the pandemic form of HIV-1 (or M group) has infected at least 60 million people and caused at least 25 million deaths. Developing countries have suffered the greatest HIV/AIDS burden, with the highest prevalence occurring in sub-Saharan Africa (2). However, enormous progress has been made in combating all facets of the epidemic including HIV-1 treatment, prevention, and cure.

Antiretroviral therapy (ART) has drastically reduced AIDS-related mortality, but does not cure HIV-1 infection. For that reason, effective HIV-1 prevention strategies are still needed. HIV-1 pre-exposure prophylaxis (PrEP) represents a new frontier in combating the HIV-1 epidemic. The antiretroviral drugs tenofovir disoproxil fumarate (TDF) and emtricitabine are

currently approved for use as PrEP as a single tablet taken daily (3). For highly adherent patients, TDF/emtricitabine PrEP has been shown to reduce HIV transmission by 86% in a cohort of MSM and by 75% in heterosexual men and women, compared to placebo (4, 5). In another study, none of the MSM who had drug concentrations in plasma consistent with daily use of PrEP became infected with HIV (6). In order to increase adherence, long acting formulations of these therapies are currently undergoing clinical trials (7).

Despite recent progress in long-acting preventatives, the prospects for an efficacious HIV-1 vaccine remain uncertain. HIV-1 vaccine design is complicated by the extreme diversity of the virus, a lack of adequate correlates of protection or viral control or clearance, and the integration of latent virus into the host genome. To date, only one human clinical trial has shown even modest efficacy against HIV-1. This trial, known as RV144, tested the efficacy of a recombinant canarypox vector vaccine plus two booster injections of a recombinant glycoprotein 120 subunit vaccine in primarily heterosexual men and women in Thailand. In the intention-to-treat analysis, there was a trend toward efficacy (p=0.08, with a vaccine efficacy of 26.4%), but in the modified per-protocol analysis (excluding 7 subjects who were found to have had HIV-1 at baseline), the vaccine efficacy was 31.2%, with p=0.04 (8). Interestingly, the vaccinated group had smaller predicted *gag* epitope repertoires than the placebo group, indicating that there was no strong impact of vaccine-induced T cell responses in this trial (9). Based on the promising results for this trial, a trial using the same canarypox vector and glycoprotein boost (HVTN 702) was attempted in South Africa but recently halted due to lack of efficacy (10).

Despite these major efforts from the global scientific community, HIV/AIDS remains a major public health crisis with 1.7 million new infections occurring annually and 770,000 individuals dying from AIDS-related illnesses. Access to treatment is far from universal. As of

2018, there were 37.9 million people living with HIV-1 but only 62% of those were accessing ART (2). These figures shed light upon the fact that a preventative vaccine is desperately needed. However, many key questions remain unanswered and many sectors of the at-risk or infected population remain underserved. The work herein focuses on the basic mechanisms of HIV-1 pathogenesis and viral control in an ART-naïve population.

Pathogenic Mechanisms of HIV-1 Infection

HIV-1 can spread by sexual, percutaneous, and perinatal routes. However, 80% of adults acquire HIV-1 following sexual exposure at mucosal surfaces (11). We will focus here on the heterosexual transmission of HIV-1. In sexual transmission events, infection is established when HIV-1 encounters a susceptible target cell in the genital mucosa where local inflammation in response to initial infection then recruits additional target cells.

HIV-1 encodes multiple pathogen-associated molecular patterns (PAMPs) that can be recognized by nucleic acid sensors of the Toll-like receptor (TLR) family, as well as intracellular PRRs including retinoic acid-inducible gene-I (RIG-I), interferon (IFN)-inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) (12, 13). Among the TLR family, TLR3, 7, 8, and 9 which are confined to the endosomes have been described to sense several nucleic acid intermediates generated during the viral life cycle. TLR3 is activated by double-stranded RNA, TLR7 by ssRNA and short double-stranded RNA, and TLR8 detects short ssRNA and ssRNA breakdown products (14). Intracellularly, HIV-1 ssRNA can be recognized by TLR7 in plasmacytoid dendritic cells (pDCs) and TLR8 in monocytes and XCR1^{+} dendritic cells. Ligand engagement of TLRs subsequently leads to the activation of proinflammatory and antimicrobial

responses via pathways involving AP-1, NF-κB, and IFN regulatory factor (IRF) 3, IRF5, and IRF7 (15).

Continued viral replication results in acute plasma viremia, which can reach several million copies of viral RNA per milliliter of blood (16, 17). Acute viremia leads to complete depletion of $CD4^+$ T cells in the gut and transient depletion of $CD4^+$ T cells in the periphery (5). Acute viremia is reduced by the induction of the cellular immune response wherein a stable set point viral load (SPVL) is reached due to the killing of infected cells by $CD8^+$ T cells (18). SPVL has long been recognized as an important predictor of disease progression, with higher SPVL predicting faster disease progression (19, 20). At this stage in infection, $CD4^+$ T cell counts rebound in the periphery, but steadily decline throughout the course of infection due to the immune system's inability to completely control viral replication.

HIV-associated inflammation is one of the most unequivocally clear consequences of HIV-1 infection and is also strongly associated with disease progression, morbidities and mortality, even in the context of ART (21). HIV-1 itself can directly induce inflammation via stimulation of immune cells and induction of both innate and adaptive arms of the immune system (22). Following engagement of viral sensors, pro-inflammatory cytokine responses are activated via the NF-κB family of transcription factors. Simultaneously, TLR engagement also activates members of the IRF family to produce the antiviral type I IFNs, IFNα and IFNβ.

Differences in the expression profile of type I IFN pathways of both $CD4^+$ and $CD8^+$ T cells have been recorded beginning in acute and chronic infection, suggesting that a stereotypical HIV-1-induced IFN pattern of gene expression in T cells might be established early in the infection and persist for years thereafter. Type I IFNs engage in a number of antiviral activities including degradation of RNA, arrest of cell cycle progression, promotion of antigen

presentation, and induction of apoptosis in infected cells (23). These activities are promoted through stimulation of the production of ISGs including SAMHD1, MX2, APOBEC3G, TRIM22, TRIM14, ISG15, and Tetherin (24). However, they simultaneously contribute to the induction of chronic immune activation (25). This is evident in the rhesus macaque model, where IFNα administration was shown to initially upregulate expression of IFN-stimulated genes (ISGs) and prevent systemic SIV infection. However, continued IFNα treatment also resulted in accelerated $CD4^+$ T cell depletion and increased viremia (26). In agreement with the results obtained from the primate models of SIV infection, several studies have reported a strong activation of different components associated with the type I IFN response (including IRFs, ISGs and viral DNA sensors) during chronic HIV-1 infection (27-31). High levels of ISGs such as IP-10 are also associated with more rapid CD4+ T cell depletion (32, 33).

Further inflammation during infection occurs via indirect or ‘bystander’ mechanisms, due to factors such as microbial translocation, cytomegalovirus (CMV) reactivation, and other mechanisms, which can both cause and be an effect of $CD4^+$ T cell depletion (34). The precise mechanism by which HIV-1 causes progressive depletion of $CD4^+$ T cells remains debated with both direct and indirect cytopathic effects proposed. When immortalized T cell lines are infected with laboratory-adapted HIV-1 strains, direct $CD4^+$ T cell killing predominates. Conversely, in more physiological systems, such as infection of lymphoid tissue with primary HIV-1 isolates, the majority of dying cells appear as uninfected “bystander” CD4 T^+ cells (35, 36). In 2010, Doitsch *et al*. demonstrated in human lymphoid aggregated cultures (HLACs) that caspase-1 driven pyroptosis results in the spilling of cytosolic contents, containing highly inflammatory cytokines, namely IL-1β (37).

Continued $CD4^+$ T cell killing ultimately leads to AIDS, which is clinically defined as less than 200 $CD4^+$ T cells per mm^3, and the acquisition of opportunistic infections (20). The majority of individuals infected with HIV-1 will progress to AIDS in the absence of ART, although they do so at varying rates. In fact, some individuals, termed long term non-progressors (LTNP) maintain healthy $CD4^+$ T cell counts and exhibit low-level viral replication for many years following infection. Within this group are individuals termed elite controllers (EC), who control viral replication below detectable levels in the absence of ART. An intermediate group of viremic controllers (VC) have the ability to suppress their viral load (VL) to low levels, though they may have high or low $CD4^+$ cell counts (38). The existence of these rare populations suggests that progression to AIDS may not be an inescapable outcome of HIV-1 infection and that there are immunologic and virologic factors that have the potential to limit viral replication and protect $CD4^+$ T cells in infected persons.

HIV/AIDS in Women

Although AIDS was first identified as a disease affecting homosexual men, it is now clear that this disease affects both men and women, and can be transmitted through both homosexual and heterosexual contact. Many studies of transmission and pathogenesis have utilized cohorts of men who have sex with men (MSM), offering little insight into the unique aspects of HIV-1 infection and disease trajectory in women. The most common mode of transmission to women is through sexual contact with a male partner (87% in the U.S.). Recent estimates suggest that women make up 25% of new HIV-1 diagnoses of adolescents and adults, and represent 20% of all AIDS diagnoses since the beginning of the epidemic in the United States. Among these diagnoses, African American women were significantly overrepresented

(62% of new diagnoses). However in sub-Saharan Africa, which represents the majority of global infections, women are over represented among infected persons, accounting for 58% of all infections (39). A combination of social and epidemiological factors contribute to this extremely high risk.

Beginning from the point of transmission, there are sex-specific differences that impact HIV-1 acquisition and pathogenesis. Women are twice as likely to acquire HIV-1 from men during sexual intercourse than men are from women. The per-exposure female-to-male transmission rate of HIV is estimated at approximately 1 in 700 to 1 in 3,000. However, the per-exposure male-to-female transmission rate is significantly higher, at 1 in 200 to 1 in 2,000 (40). Interestingly, increased female susceptibility to *in utero* HIV-1 transmission has also been observed. In several cohorts of *in utero* infected infants, infected females significantly outnumber males (41-44). A recent study by Adland *et al.* proposes a mechanism by which increased immune activation (HLA-DR$^+$ CD4$^+$ T cells) in cells of female fetuses provides additional targets for infection (44). The observation of this phenomenon of increased female susceptibility to infection over multiple routes of transmission and in both adults and in fetuses suggests there are likely a wide range of immunological and other biological explanations.

The major routes for heterosexual transmission of HIV-1 to adult women include the vagina and other parts of the female genital tract (FGT) in women, and the penis/foreskin in men, which constitute parts of the genital mucosa. Mucosal barriers, and in particular the genital mucosa, constitute a critical barrier between the external environment and the human body. Several components influence the effectiveness of the mucosal surface as a barrier against HIV acquisition, including the physical anatomy, cellular immunity, and the microbiome. Barrier

damage, inflammation, and altered microbiome composition can have an effect not just on HIV-1 acquisition but also disease progression (6).

The FGT is protected from virus by many layers of squamous epithelium in the ectocervix and vagina and by a single layer of columnar epithelium in the endocervix. The FGT also contains a layer of flattened epithelial cells called the stratum corneum which provides a further barrier. Epithelial integrity at all sites is facilitated by protein structures that adhere cells to one another or to the extracellular matrix. Penetration of HIV-1 into the squamous epithelium can be significantly enhanced by tight junction interruption (45).

In addition to the cellular barrier, a layer of secreted mucus is also essential to host defense. The mucus contains soluble proteins that provide immune defense and anti-inflammatory protection against epithelial damage. The mucus itself also provides a physical barrier against virus penetration. For HIV-1 transmission to occur, infectious virions or infected donor cells must cross these barriers and locate a susceptible host cell (46). Though the exact sites of infection in humans are unclear, an elegant study by Steih, *et al*. demonstrated using an SIV-dual reporter system in rhesus macaques, that SIV infection can occur at several sites throughout the upper and lower FGT. Using a high dose of a single round non-replicating SIV-based vector, they showed evidence of transduced cells throughout the entire FGT, including the vagina, ecto- and endocervix, along with ovaries and local draining lymph nodes after only 48 hours. This wide distribution demonstrates that virions quickly disseminate after exposure to target cells throughout the FGT, although with a preference for infection in squamous vaginal and ectocervical mucosa. In this study, $CD4^+$ T cells resident throughout the FGT were the primary target cells (47).

An inflammatory cascade at the mucosal site is further required for the establishment of productive viral infection (48). In contrast to many other viral or bacterial infections, increases in inflammatory responses are actually associated with increased risk of infection. A wide range of pro-inflammatory products with diverse immune functions have been implicated in risk of HIV-1 acquisition including a-defensins, MIP-1α, IL-8, MIP-1β, IL-1β, IL-1α, and TNF-α (49). The effects of these products are wide-ranging, and proteomic analysis by Arnold and Burgener *et al*. demonstrated this pro-inflammatory cytokine profile is linked with increased neutrophil protease levels, barrier disruption, and increased frequency of cervicovaginal $CD4^+$ T cells in women (50). Rather than providing protection from infection, the increase in activated target cells aids the establishment of HIV-1 infection.

The vaginal microbiome is another critical element in protection from infection. As such, changes in vaginal microbial communities are consistently associated with increased HIV-1 risk. This is exemplified by a striking increase in HIV-1 susceptibility with bacterial vaginosis (BV) by as much as 60% (15). BV occurs when protective microbiota in the vagina, dominated by lactic acid-producing Lactobacillus species which are associated with protection from HIV transmission, are replaced by diverse strains of bacteria such as Gardnerella, Atopobium, Prevotella, Fusobacterium spp., and other BV-associated bacteria (BVAB) (16). BV is associated with increased pro-inflammatory cytokine levels, particularly IL-1β (17). Some BVAB are associated with cervicitis in humans which may induce recruitment of T cells, and treatment of BV with metronidazole led to decreases in mucosal $CCR5^+CD4^+$ T cells (18). A study by Anahtar *et al*. demonstrated that high ecological diversity of FGT microflora drives enhanced stimulation of TLR4 and NFκB, leading to increased pro-inflammatory cytokines and a subsequent increase in activated $CCR5^+CD4^+$ T cells which can become targets for HIV

infection (19). Studies of the mucosal proteome during BV demonstrated lower levels of factors important for an effective physical barrier including small proline-rich proteins and involucrin (51). Thus, it is possible that microflora diversity may be an underlying component and/or driver of host inflammation responses and HIV-1 acquisition.

Relatedly, concurrent sexually transmitted infections (STIs) also have a strong epidemiological link with increased risk of HIV-1 acquisition. One of the best-described STIs that increases HIV-1 risk is Herpes simplex virus 2 (HSV-2) (52). While the mechanism by which this occurs remains unclear, one hypothesis is that innate host pro/anti-inflammatory mediators (including those detailed above) modulate HIV-1 susceptibility (53). Indeed, a higher pro-inflammatory profile characterized by increased numbers of HIV-1 target cells upon HSV-2 infection can persist in mucosal tissues (54). HSV-2 also contributes to the amplification of target cells expressing homing markers (α4β7) in the absence of viral shedding and increased activated $CD4^{+}CCR5^{+}$ T cells (and subsequently higher HIV-1 infectivity), as demonstrated in female genital tract (FGT) mucosal explant tissue (55). Conversely, reduced inflammation and immune activation at mucosal surfaces are associated with HIV-1 protection. Decreased immune activation, characterized by reduced systemic $CD4^{+}$ T cell gene expression coupled with lowered mucosal cytokine/chemokine expression (including MIG, IP-10, and IL-1α) may collectively limit target cell availability and activation and hence reduced risk of infection (56).

Exposure to semen during sexual intercourse may also affect HIV-1 susceptibility in the FGT. Semen itself is highly basic and increases vaginal pH (normally kept low by the production of lactic acid by Lactobacillus), which may in turn alter the protective mucus layer in the FGT as well as the microbiome (57). Perhaps as a result of these alterations, semen exposure increases pro-inflammatory cytokines in the FGT (such as IL-6, IL-8 and IL-1β) compared to protected

coitus or abstinence, and is associated with increased $CD4^+$ T cells and macrophages shortly after coitus (58). As detailed above, increased inflammatory cytokine levels in the genital mucosa, and the associated recruitment of activated target cells, are a significant risk factor for HIV-1 acquisition.

Hormone levels may also play a role in HIV-1 acquisition. Studies in rhesus macaques have demonstrated increased susceptibility to SIV infection during the luteal phase of the menstrual cycle (which is characterized by high progesterone levels) when compared to the follicular phase (characterized by high estrogen levels). Importantly, injectable contraceptives, which tend to be progesterone or progestin-based, have also been associated with increased HIV infection risk in some, but not all studies. A meta-analysis by Ralph, *et al*. found evidence of increased acquisition risk in in ten of twelve studies of women using depot medroxyprogesterone acetate (DMPA), but no evidence of increased risk in ten studies of oral contraceptive pills (59). Use of these contraceptives may affect HIV-1 target cell levels or host innate immune factors. Women using injectable DMPA had increased concentration of several soluble proteins of the innate and adaptive immune system, including IL-1α, IL-1β, IL-2, MIP-1β, IP-10, IL-8, TGF-β, HBD4, IgA, IgG1, and IgG2 (60). Conversely, Wall, *et al*. found no effect of hormonal contraceptive use on HIV-1 acquisition in Zambian serodiscordant couples (61). However, consideration of the effects of these hormonal variables is important, as family planning and HIV-1 prevention interventions often go hand in hand.

In a 2014 study of HIV-1 transmission dynamics, Carlson *et al.* demonstrated that in the context of HIV-1 infection, sex represents a host factor that may also influence the characteristics of the transmitted virus. In this study, there was a significant bias for the transmission of consensus-like residues. However, a significantly weaker bias in male-to-female

transmission was observed than in female-to-male transmission. Therefore, women became infected with viruses that were significantly less consensus-like than those transmitted to men. The mitigation of the transmission bottleneck in male-to-female transmission was comparable to female-to-male transmission where the receiving partner exhibited genital ulcers or inflammation (62). The abovementioned report regarding *in utero* HIV-1 infection found additionally that female fetuses were infected with viruses that on average exhibited less replication capacity and increased IFN resistance than those transmitted in male fetuses (44).

Sex and the Immune Response

A growing body of research now demonstrates that women show higher humoral and innate immune responses to infection or other antigenic stimulation. In a variety of settings, women have been shown to exhibit more robust immune responses to both primary viral infections as well as vaccination when compared with men (63). The mechanisms underlying the observed increased immune function in women are not yet fully characterized, but evidence suggests that there are genetic as well as hormonal determinants. These differential immune responses are often attributed to the biased response from the X chromosome. The human X chromosome encodes a number of genes that are critical to the immune response including TLR7 and 8, FOXP3 (the master transcriptional regulator of regulatory $CD4^+$ T cells, and CTLA-4 (a T cell co-inhibitory molecule). The X chromosome also codes for a number of micro RNAs that regulate immune cell function including miR-233, miR-106A, and miR-424 (64). Interestingly, a number of these encoded micro RNAs have been shown to downregulate negative regulators of the immune system such as FOXP3 and CTLA-4 (65).

Females carry two X chromosomes while males have only one. However, female mammals undergo a process of X chromosome inactivation (XCI) whereby one X chromosome is transcriptionally silenced. This leads to cellular mosaicism, meaning that the X chromosome of either maternal or paternal origin may be expressed in different cell populations. This has proven to be an immunological advantage for females, as an X-linked gene mutation will only be expressed in some cells, whereas the mutation will be expressed in all cells in males. As a result, many diseases resulting from mutations of the X chromosome, such as X-linked severe combined immunodeficiency (SCID), are more prevalent in males (66). And conversely, mosaicism of the X chromosome equips females with a more diversified immune response which is better equipped for antigen recognition. Interestingly, however, the process of XCI is not always uniform. Souyris, *et al.* recently demonstrated that the TLR7 gene is able to escape XCI in pDCs, B cells, and monocytes. They show biallelic expression of TLR7 in all donors tested (women and XXY males), with frequencies ranging from 7% to 45% (67).

Another factor that may contribute to sex differences in the immune response are differences in the TLR responses that are involved in the recognition of viral and bacterial pathogens. Recent studies demonstrate that exposure of peripheral blood mononuclear cells (PBMCs) to TLR7 ligands *in vitro* causes higher production of IFNα in cells from women than from men (68, 69). Compared to women, PBMCs from men produced not only lower amounts of IFNα in response to TLR7 ligands but also higher amounts of the immunosuppressive cytokine IL-10 after stimulation with TLR8 and TLR9 ligands or viruses. Interestingly, IL-10 production after TLR9 ligand or HSV-1 stimulation was significantly related with plasma levels of sex hormones in both groups, whereas no correlation was found in cytokines produced following TLR7 and TLR8 stimulation (70). Further transcriptional analyses revealed sex differences in the

expression of genes along TLR pathways and induction of type I IFN responses. Following either vaccination in adult humans or virus challenge in adult rats, the expression of TLR-pathway and pro-inflammatory genes (for example, TLR7, MYD88, RIGI, IRF7, IFNB, JAK2, STAT3, NFKB, IFNG, and TNF) is higher in female than male PBMCs from humans as well as in tissues from female rats (71).

While the primary focus of this work is the innate cellular immune response, women also exhibit more robust humoral immune responses. At baseline, immunoglobulin levels and B cell numbers tend to be higher in women than men (72). Women additionally exhibit more active humoral immune responses to vaccination when compared with men (63, 65). For example, a cross- sectional study of children over 15 years of age, who received the MMR vaccine at 12–15 months after birth, indicated that the prevalence of serum IgG antibodies against measles, mumps, and rubella was significantly higher in girls than boys (73). Moreover, adults 18–49 years of age exhibited HAI antibody titers that were at least twice as high in females compared to males following receipt of either a full or half dose of seasonal trivalent influenza vaccine (74).

As one might expect, this enhanced immune activation is additionally interconnected with exacerbated immunopathology and autoimmune disease (37). In fact, conservative studies estimate that 78% of individuals with autoimmune diseases are women. In some instances this phenomenon is related to the increased humoral immune responses found in women, whereby the increased levels of antibodies in serum protects women from infection, but simultaneously increased levels of autoantibodies contribute to higher levels of autoimmunity (75). The profound sex bias observed in systemic lupus erythematosus (SLE) is additionally attributed to an “IFN signature” resulting from the increased activation of type I IFN signaling observed in women (76).

Hormonal differences between men and women also contribute to their demonstrably different immune responses. Immune cells including lymphocytes and myeloid cells express estrogen, androgen (testosterone), and progesterone receptors. Androgens are hormones that stimulate or control the development and maintenance of male sex characteristics in vertebrates, although they are present in both males and females to varying degrees. Androgens are generally anti-inflammatory. For example, surface expression of TLR4 on macrophages is reduced by exposure to testosterone both *in vitro* and *in vivo* in mice (77). Testosterone has also been demonstrated to increase the production of IL-10 and transforming growth factor-β (TGFβ) synthesis by $CD4^+$ T cells (78). In humans, androgen deficiency in men has been reported to induce increased levels of inflammatory cytokines (IL-1β, IL-2, and TNF) as well as $CD4^+$/$CD8^+$ T cell ratio and antibodies titers (79, 80). Several studies have indicated that androgens exert an overall inhibitory effect on differentiation of T_H1 arm of the immune system, with consequent reduced production of IFNγ (81, 82).

Progesterone also has broad anti-inflammatory effects. In animal models, progesterone-exposed macrophages and dendritic cells (DCs) produce lower amounts of IL-1β and TNF compared with untreated cells (83, 84). Treatment of human NK cells with progesterone reduces cellular activation and production of IFNγ via caspase-dependent apoptosis (85). Progesterone can additionally promote skewing of $CD4^+$ T cell responses from T_H1-type towards T_H2-type responses, as characterized by increased IL-4, IL-5, and IL-10 production (86).

This work will focus primarily on estrogen, which exerts its functions by binding to the estrogen receptors ERα and ERβ. Immune cells including B cells, T cells, and DCs express ERs. $CD4^+$ T cells express higher levels of ERα than ERβ, whereas B cells exhibit the opposite pattern and express higher levels of ERβ than ERα. Only very low but comparable levels of both

receptors were detected in $CD8^+$ T cells (87). ERs are ligand dependent transcription factors that form complexes at specific DNA sites with chromatin modifying co-regulators and other transcription factors, leading to epigenetic modifications of chromatin and transcription initiation (88, 89). Once in the nucleus, ERα is preferentially recruited to estrogen response elements (EREs) present in open chromatin, where it binds directly or is tethered indirectly in complexes with other transcription factors. These include factors such as NF-KB, SP1, AP-1, C/EBPß that are important in immune cell function (90, 91). Putative EREs are present in the promoters of several innate immunity genes including MyD88, Mx2, and IFNAR1, suggesting that sex steroid hormones may directly cause dimorphic innate immune responses (71).

Although classical actions of estrogen are mediated via a transcriptional, or genomic, pathway, it was recently recognized that estrogen can also elicit rapid biologic effects via a nongenomic pathway (92, 93). This pathway is mediated by plasma membrane associated ERα and possibly other estrogen binding signaling molecules. Estrogen stimulation leads to the recruitment of the membrane associated ERα into multiprotein signaling complexes. One of the critical signaling molecules in these estrogen-induced complexes is the cellular proto-oncogene Src. This kinase associates with ERα upon estrogen stimulation and is critical for the estrogen-induced activation of downstream effectors such as MAPK and Akt. Although these noncanonical effects of estrogen were initially described in tissues such as breast and vasculature, T cells have been reported to contain putative plasma membrane receptors for estrogen that can regulate early signaling events via a nongenomic pathway and can modify the responsiveness of T cells to T cell receptor engagement (94).

There are 4 forms of estrogen: estrone (E1), estriol (E3), estetrol (E4), and estradiol (E2); the latter is the most abundant in pre-menopausal non-pregnant women and also has the greatest

affinity for ERα (95). For that reason, this work will focus primarily on the functions of E2, which has been shown to increase a variety of humoral and cell mediated immune responses. For example, E2 positively regulates TLR-mediated pro-inflammatory pathways in murine macrophages and pDCs (96-98). In addition, pDCs from female humans and mice have higher basal levels of IFN regulatory factor 5 (IRF5) and IFNα production following TLR7 ligand stimulation, and transcriptional regulation of IRF5 in female mice was found to be under the control of signaling through ERα (99).

However, there is also some research which indicates that many of the direct effects of estradiol on $CD4^+$ T cells are anti-inflammatory, with evidence to suggest that estradiol inhibits expression of the IL-2 receptor and promotes the suppressive function of regulatory $CD4^+$ T cells (100-105). As discussed previously in the context of pDCs from women producing higher levels of both IFNα and IL-10, induction of an inflammatory response and anti-inflammatory counter response often go hand in hand.

Many studies have shown that estrogen can influence T_H1 and T_H2 responses. Conflicting outcomes have been obtained by different groups, however, with some studies showing an enhancement of T_H1 responses but other studies demonstrating an increase in T_H2 responses. Some of the variability observed in the different studies is believed to be due to the fact that estrogen may exert a biphasic effect on T_H1/T_H2 differentiation. Specifically, low doses of estrogen reportedly promote enhanced T_H1 responses and increased cell-mediated immunity (106-108). Exposure of T cells to E2 also enhances their ability to respond to IL-12, a classic T_H1 cytokine, by augmenting the activation of Stat4 which is a crucial component of the IL-12 signaling pathway. E2 administration has also been found to augment the expression of T-bet (a

master regulator of T_H1 differentiation) upon exposure of T cells to IL-27, which is an IL-12-related cytokine that also promotes T_H1 responses (109).

Sex Differences in the Immune Response to HIV-1 Infection

It is apparent that the effect of sex continues after the transmission of HIV-1 and continues to affect viral pathogenesis. Importantly, greater viral control of HIV-1 infection in women has been well documented in the literature. One of the first reports of lower plasma VL in women was a 1997 study by Evans, *et al*. wherein VL was assessed in a cohort of 28 men and 14 women at three equally dispersed time points over a mean of 43 months. At these three time points, median VL was 3.3-, 4.9-, and 1.5 fold lower, respectively, in women than in men. Interestingly, despite these differences in VL, the authors found no sex differences in the relative risk of developing AIDS-defining end points or death (110).

A 1998 study in larger cohort of 650 injection drug users (IDU) also found lower median VL in women than in men (45,416 vs. 93,130 copies/mL) when measured by qPCR (consistent with Evans, *et al*.). The association between sex and VL remained significant even after adjustment for $CD4^+$ cell count, race, and drug use within the previous 6 months. As in Evans*, et al*., time to AIDS was statistically similar for men and women in a univariate proportional-hazards model and in a model adjusting for $CD4^+$ cell count. Proportional-hazards models showed that women with the same VL as men had a 1.6-fold higher risk of AIDS; or, equivalently, that women with half the VL of men had a similar time to AIDS as men (111).

Both studies mentioned above were limited by their inability to account for duration of infection, and may have been affected by individuals in different stages of infection. However, their results were recapitulated in a nested case-control study of 24 IDU, wherein initial median

VL in female patients was 14,918 copies/mL compared to 148,354 copies/mL in male patients. VL then increased more rapidly in women than in men and subsequently converged (112). (69, 113). This control of viremia has not been associated with a significant benefit in survival, either in terms of $CD4^+$ T cell decline or time to AIDS (111, 112, 114-116).

Recent studies aimed at further understanding the mechanism(s) underlying these disparate clinical parameters have demonstrated that chronically infected women initiate stronger innate immune responses to HIV-1 infection. These responses are characterized by increased expression of IFN-stimulated genes (ISGs), which are, in turn, linked to increased cellular immune activation (69, 117). Chang, *et al*. isolated T cell and pDC populations from ART-naïve, chronically infected individuals and assessed the expression of 98 genes involved in TLR and type I IFN signaling pathways. Several ISGs were significantly correlated with HIV-1 VL and/or $CD4^+$ T cell count. Higher expression levels of a subset of these ISGs, including ISG15, MX1, and CCR5 were observed in cells derived from women as compared to males after adjusting for VL and were correlated to higher levels of T cell activation. These data show that higher IFNα production is associated with higher *ex vivo* expression of several ISGs in women.

A study by Meier, *et al*. demonstrated higher percentages of $HLA\text{-}DR^+CD38^+$ $CD8^+$ T cells in chronically infected women prior to ART initiation when compared to men. Increased levels $HLA\text{-}DR^+CD38^+$ $CD8^+$ T cells have been consistently linked with faster disease progression in HIV-infected individuals. Furthermore, the same study showed that pDCs derived from women produced more IFNα in response to TLR-7 stimulation with equivalent amounts of HIV-1 RNA (69). These data show that sex differences in TLR-mediated activation of pDCs may account for higher immune activation in women compared to men at a given HIV-1 VL.

A handful of studies have linked sex hormones to differences in HIV pathogenesis between men and women (118-123). Several of these reports describe inhibition of HIV-1 replication by estrogen, but some report no effect or even enhancement of replication. However, direct comparison of these studies is complicated by differences in concentration of estrogen, cell type and donor sex, HIV-1 strain, and inclusion of additional sex steroid hormones in the experimental design. For example, a study of HIV-1 replication in PBMC with both estrogen and progesterone finds mid-proliferative phase conditions (low concentrations of both hormones) increased, and mid-secretory (high concentrations of both hormones) phase conditions decreased, HIV-1 replication (118). However, this does not address the individual effects of each hormone, which are known have distinct physiological functions (124, 125). Another study performed in PBMC from male and female donors found that, in the absence of physiological concentrations of hormone treatment, virus replication could be stratified by HIV-1 subtypes, with subtypes B and C replicating more highly in these conditions than subtypes A and D. While the dose of sex steroid hormone treatment influenced HIV replication and transmission kinetics, ad such effects were inconsistent between donors and HIV subtypes (123).

Furthermore, the mechanism behind a potential inhibition of HIV-1 replication by estrogen is still a matter of debate. Tasker, *et al.* report that while E2 protects primary macrophages against HIV-1 infection via an entry-related mechanism, E2 did not affect surface expression of CD4 and HIV co-receptors nor HIV attachment. Rather, they credit the induction of IFNα with restricting viral replication. While they observe E2 upregulated gene expression of ISGs in monocyte derived macrophages (MDMs) from multiple donors, induction of host restriction factors APOBEC3G, APOBEC3F, or SAMHD1 was not consistent, with exception of APOBEC3A (119).

Conversely, Devadas *et al.* report the downregulation of CXCL9, CXCL10 and CXCL11 chemokines and IL-1β, IL-6 cytokines in MDMs infected with HIV-1 pre-treated with high concentrations of E2 or progesterone compared to untreated HIV-1 infected cells or HIV-1 infected cells treated with low concentrations of E2 or progesterone (120). However, the high concentrations of E2 used (in the micromolar range) better reflect human pregnancy levels than average physiological concentrations. Alternatively, Szotek, *et al*. describe a mechanism by which E2 inhibits HIV-1 replication by inducing a complex formation between β-Catenin and ERα on the HIV-1 promoter to suppress transcription (121).

A recent report on as ERα as a regulator of HIV-1 latency reports a similar mechanism by which the receptor reduces reactivation of HIV-1 from latency (126). In this study, selective estrogen receptor modulators (SERM) were used as proviral activators that sensitized cells to latency-reversing agents (LRA) while E2 inhibited HIV transcription. The authors reported that E2 treatment of $CD4^+$ T cells in an *in vitro* latency model reduced reactivation of latent virus, by inducing higher levels of its receptor which bound to the HIV-1 long terminal repeat (LTR) region and suppressed viral transcription directly. Interestingly, they additionally report in a small number of individuals that this phenomenon may cause the inducible reservoir to be smaller in women. It remains to be seen whether the smaller reservoir size in women is a direct consequence of the inhibition of HIV-1 reactivation and replication by E2, whether better immunological control by women leads to a reduction in the reservoir size, or whether a combination of both factors is required.

Interestingly, Adland, *et al.* report that the proviral DNA burden in male children infected with HIV-1 *in utero* is smaller than that in female children and that boys are more likely to remain virally suppressed during ART interruption (44). The observations of Das and Adland are

not necessarily in conflict as there have often been significant differences observed between adult and pediatric HIV-1 infection (127, 128). The authors of this study propose a mechanism by which female fetuses are preferentially infected due to increased activation of $CD4^+$ T cells. They further argue that the viruses transmitted to male fetuses are on average more IFN-sensitive and therefore more subject to immune control in the context of ART interruption (44).

While there are very few studies looking at post-menopausal women infected with HIV-1, one recent study suggested that geriatric women were actually less likely to remain suppressed on ART than men (129). Although the conditions are different, this would be in contrast to data in younger women which finds that women in many populations are more likely to suppress virus spontaneously, or maintain relative control compared to men. If these observations are correct, there are a number of potential explanations. Importantly, the mechanisms of viral suppression on ART may be sufficiently different from those required for relative control of viremia that we and others observe in untreated women. Recent data suggests that $CD8^+$ T cells play a critical role in viral suppression on ART via both cytolytic and non-cytolytic mechanisms (130, 131). Very little is known about sex differences in either general or HIV-specific $CD8^+$ T cell responses (69). Hormone levels may also play a role in these observations, as both geriatric patients and children or fetuses would have significant differences in hormone levels than the reproductive-age women studied here. *In utero,* fetuses of both sexes are exposed to high levels of maternal and placental estrogens and progesterone. However, testosterone is produced by males from 8-14 weeks of gestation (132). Therefore, it is reasonable to hypothesize that differential immune regulation by sex hormones play a role in these paradoxical observations.

Despite the accumulating immunologic data describing the sex differences inherent to chronic HIV-1 infection, much remains to be elucidated with regard to the acute stages of

infection. The preponderance of data generated to date relies on samples derived from cross-sectional cohorts, during the chronic stages of infection, with limited longitudinal follow up. The potential immunological disparities between men and women during the acute stages of infection, which undoubtedly set the stage for later pathogenesis, are clearly understudied. This work, therefore, aims to define sex-specific differences in HIV-1 pathogenesis and the impact of inflammation on early immune responses and disease progression.

While there have been great advances in the field of HIV-1 research, many basic questions surrounding the pathogenesis of HIV-1 still remain. The well-established paradigm of lower VL in infected women provides the impetus to study in greater detail the potential differences in HIV-1 pathogenesis between men and women. This will not only benefit public health interventions designed specifically for cisgender and transgender women, but will also help elucidate the mechanisms behind control of viremia and $CD4^+$ T cell loss. Here we describe studies in a population of heterosexual Zambian men and women in the absence of ART beginning in acute HIV-1 infection and continuing over 30 months. We compare disease course, $CD4^+$ T activation, and the effect of sex hormones in these individuals. We further describe studies to elucidate the transcriptional differences in the $CD4^+$ T cells of these individuals during acute infection that set the stage for the immune response throughout infection.

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Chapter II: Better viral control despite higher $CD4^+$ T cell activation during acute HIV-1 infection in Zambian women is linked to the sex hormone estradiol

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Abstract

The influence of biological sex on disease progression in HIV-1 infected individuals has been focused on the chronic stage of infection, but little is known about how sex differences influence acute HIV-1 infection. We observed profound differences in viral load and $CD4^+$ T cell activation from the earliest time-points in men and women in a Zambian heterosexual acute infection cohort. Women exhibited a more than two-fold higher rate of $CD4^+$ T cell loss despite significantly lower viral loads (VL) than men. The importance of studying acute infection was highlighted by the observation that very early in infection, women exhibited significantly higher levels of $CD4^+$ T cell activation, a difference that was lost over the first 3 years of infection as activation in men increased. In women, activation of $CD4^+$ T cells in the acute phase was significantly correlated to plasma levels of 17β-estradiol (E2). However, unlike in men, higher $CD4^+$ T cell activation in women was not associated with higher VL. In contrast, a higher E2 level in early infection was associated with lower early and set-point VL in women. We attribute this to an inhibitory effect of estradiol on virus replication, which we were able to observe with relevant transmitted/founder viruses *in vitro*. Thus, estradiol plays a key role in defining major differences between men and women during early HIV-1 infection by contributing to both viral control and $CD4^+$ T cell loss, an effect that extends into the chronic phase of the disease.

Introduction

Although sex differences in response to pathogenic infections have been well documented in the literature (1-3), many studies of HIV-1 transmission and pathogenesis to date utilize single cohorts (commonly men who have sex with men (MSM) or female sex workers) or cohorts that are significantly biased towards one sex and are thus unable to directly compare disease course and outcomes between men and women. As a result, robust studies comparing how the immune response to HIV-1 differs between men and women, particularly during the early phase of infection, have been difficult to perform. Current estimates suggest that 17.8 million women are infected with HIV-1 and that it is the leading cause of death in women of reproductive age (15-49) (4). Thus, a detailed understanding of the biology of HIV-1 infection in women is essential to combating the global HIV-1 epidemic in general.

A growing body of data has demonstrated that women exhibit more robust immune responses to both primary viral infections as well as vaccination when compared with men (2, 3). In HIV-1 specifically, several studies have reported that chronically infected women have significantly lower plasma viremia than men, although the reason for this discrepancy has yet to be identified (5-10). Additionally, a study comparing men and women chronically-infected with HIV-1 found that infected women had a higher proportion of activated ($CD38^{+}HLA\text{-}DR^{+}$) $CD8^{+}$, but not $CD4^{+}$, T cells prior to antiretroviral therapy (ART) initiation, suggesting higher levels of detrimental immune activation (11). Furthermore, several interferon-stimulated genes (ISGs) were found to be expressed at significantly higher levels in women than men during the chronic stage of HIV-1 infection, after correcting for plasma viral load, implying a more activated innate phenotype in females given similar exposure to viral antigen (12). However, existing studies are limited by their utilization of patient samples from individuals of different ethnic backgrounds, in

different geographical locations, or with unknown dates and routes of infection. The present study has focused on a cohort of Zambian, heterosexual, acutely-infected individuals, allowing for comparison of epidemiological and immunological parameters in a group that share a similar environment and genetic background. To our knowledge, these data represent the first longitudinal comparison of the immune response to natural HIV-1 infection that encompasses both acute and chronic infection of both men and women. The studies presented here demonstrate that the discrepancy in viral load observed in women is established early in infection and that $CD4^+$ T cell loss is double that of men in the first few years of infection. These differences are linked in women to estradiol levels, which correlate with $CD4^+$ T cell activation and lower viral loads.

Results

Women exhibit a faster rate of $CD4^+$ T cell decline and lower plasma viral loads early in HIV-1 infection

In order to understand the impact of biological sex on HIV-1 disease course, viral load and CD4+ T cell counts were compared in acutely individuals enrolled in the Zambia Emory HIV Research Project (ZEHRP) cohort at sites in Lusaka and Ndola, Zambia. Post-peak viral loads of male and female participants (n = 158; 72 female (F), 86 male (M)) were plotted between 30 and 900 days post-estimated date of infection (EDI). 97% of these individuals were infected with a subtype C variant. The estimated date of infection was calculated as previously described based on the date of last seronegative test, date of first seropositive test, and presence or absence of p24 antigen (13). Subjects were selected on the basis of ART-naïve status and availability of at least two viral load measurements within the time interval, including measurements within 90 days of the beginning and end of the interval. The median age of these subjects was 31 at the time of study enrollment. During this period, women had consistently lower plasma viral loads (VL) than men, as indicated by the difference in the y-intercepts but not the slopes of the linear regression line for each sex (Fig 1A).

$CD4^+$ T cell counts of a subset of 116 individuals (56 F, 60 M) were then plotted for individuals who met the aforementioned criteria and for whom $CD4^+$ cell counts were available. When the linear regression lines for $CD4^+$ T cell counts of each sex are compared, the slopes differ significantly (Fig 1B). Comparison of slopes of individual linear regression lines of $CD4^+$ cell counts over this interval indicates that women exhibit a greater than 2-fold higher rate of loss (median slope of -0.21 versus -0.10 for men, Fig 1C). Importantly, $CD4^+$ T cell counts are

significantly higher in HIV-1 uninfected women than uninfected men, as previously reported in individuals in Lusaka, Zambia (14). Thus, $CD4^+$ T cell counts initially remain higher in women than men following infection, although the rate of $CD4^+$ decline is higher in women, such that by 900 days men and women have similar numbers of $CD4^+$ T cells. Indeed, a Kaplan-Meier survival analysis of the time to a $CD4^+$ T cell count of <300/µL over the first 900 days of infection yielded curves that are not significantly different for men and women (Fig 1D), despite women starting out with higher $CD4^+$ T cells counts.

$CD4^+$ T cells are more highly activated in women during acute infection

Chronic immune activation is a hallmark of HIV-1 infection and a reliable predictor of $CD4^+$ T cell loss (15). In order to determine whether differential T cell activation contributes to faster rates of $CD4^+$ cell loss in women despite consistently lower levels of plasma viremia, markers of cellular activation on PBMC were analyzed in 47 (22 F, 25 M) HIV-1 infected individuals at 1, 9 and 30 months post-infection by flow cytometry. Subjects were randomly selected based on sample availability and ART-naïve status. All subjects were infected through heterosexual contact with HIV-1 group M subtype C viruses.

At 1-month post-EDI a greater percentage of $CD4^+$ T cells from women than men expressed the activation marker CD38 (Fig 2D). This difference was apparent in central ($CCR7^+CD27^+CD45RO^+$, Fig. 2A) and effector memory ($CCR7^-CD27^-CD45RO^+$, Fig 2B), and as well as naïve ($CCR7^+CD27^+CD45RO^-$, Fig. 2C) $CD4^+$ T cell subsets. These sex differences are still significant in the memory $CD4^+$ T cell subsets at 9 months post infection. By 30 months post-EDI there were no significant sex differences in the levels of $CD4^+$ T cell activation in the memory subsets, although there was still a significantly greater proportion of $CD38^+$ naïve $CD4^+$

T cells in women than men. Interestingly, the reduced difference during follow-up was primarily because the percentage of $CD38^+$ T cells in men increased to a level similar to that of women over time; while total $CD38^+$ $CD4^+$ T cells increased between uninfected women and women at 1 months post-EDI, it was not until the chronic stage of infection that there was a significant increase in men.

In order to understand how $CD4^+$ T cell activation affected the extent of viral replication and VL, we compared $CD4^+$ T cell activation at 1 month post-EDI with set point VL (Log_{10}SPVL calculated as geometric mean of VL from 1-12 months). In men, there was a significant positive association between total $CD4^+$ T cell activation at 1 month post-EDI and log_{10} SPVL (Fig 2C). However, there was no relationship between these parameters in women (Fig 2D), demonstrating that despite heightened $CD4^+$ T cell activation, there was no commensurate increase in VL. These data are consistent with the observation that women consistently maintain lower plasma viremia than men despite higher $CD4^+$ T cell activation.

Estradiol is associated with viral load and $CD4^+$ T cell activation

In order to address the cause of heightened T cell activation in acutely infected women, we measured estradiol (E2) in time point-matched plasma samples. While E2 and its metabolites estrone and estriol act in concert with other sex steroid hormones, E2 is the major form of estrogen found in non-pregnant pre-menopausal women in terms of absolute serum levels and affinity for estrogen receptor-α (ERα) (16, 17). It plays a role in immune signaling in $CD4^+$ T cells primarily via binding to ER-α triggering nuclear translocation and transcription from estrogen-response elements (ERE) in target genes. This impacts expression of several genes involved in the immune response including AP-1 and IFN-γ (18, 19).

We measured plasma levels of E2 by conventional ELISA at a median of 44 days post estimated date of HIV-1 infection in women and men who were included in our flow cytometry studies and additional subjects for whom VL data was available. Plasma levels of E2 were inversely correlated with time point-matched $Log_{10}VL$ in women, but not men, who had predictably lower E2 levels (Fig 3A, D). Further, E2 levels at infection predicted lower $Log_{10}SPVL$ in women, but not men (Fig B, E). Despite its association with lower levels of plasma viremia in women, we found that in women plasma E2 was positively correlated with total $CD4^+$ T cell activation at 1 month post-EDI (Fig 3C). We also measured plasma levels of progesterone in women, and based on these only 2 female subjects appeared to be in the mid-luteal phase of the menstrual cycle at the time of sampling. We did not see a significant effect of progesterone on viral load or cellular activation (Fig. 3G-I). Finally, we measured plasma levels of testosterone in order to understand whether this sex steroid hormone also played a role in HIV-1 pathogenesis. We found that there was no significant relationship between plasma levels of testosterone and $Log_{10}VL$ at the time of sample collection or $CD4^+$ T cell activation in men or women (Fig 4A-D, F). However, we did find a positive association between plasma levels of testosterone in men and $Log_{10}SPVL$ (Fig. 4E). Interestingly, this was in contrast to the negative association observed in women between E2 and $Log_{10}SPVL$.

Estradiol restricts viral replication in vitro

After observing that plasma E2 was inversely correlated with VL and SPVL in women, we sought to understand whether E2 could directly impact HIV-1 replication. Previous studies have attempted to identify the effect of sex hormones on viral replication, particularly of hepatitis C virus (HCV) and HIV-1. These studies vary in their conclusions, likely as a result of

variable methodology with respect to the dosage of E2, presence or absence of additional sex hormones, cell type and virus strain used (20-23). We assessed the *in vitro* replication of six patient-derived transmitted/founder (T/F) viruses as well as a subtype C laboratory isolate (MJ4) in the presence and absence of physiological levels of E2. Infectious molecular clones) of T/F viruses were generated as previously described, and used to infect PBMC from healthy female donors in the presence of 0, 1 or 10 nM E2 (24). PBMCs were not activated prior to infection. Virus growth was measured between 3 and 11 days post-infection and area under the curve (AUC) was calculated for each virus and compared between E2 treatment groups. Overall, we found that E2 treatment resulted in a modest but consistent restriction of viral replication. Representative replication curves for four of the T/F viruses are shown in Figure 5 A-D. Collectively, E2 treatment significantly restricted viral replication in the viruses assayed, as measured by percent AUC compared to untreated. The median replication was 84.29% of untreated at 1 nM E2 and 44.95% of untreated at 10 nM E2 (Fig 4E). A similar pattern of viral restriction of MJ4 replication by E2 was observed in 3 donors (Fig. 4F). This suggests that while E2 appears to be involved in cellular activation pathways, it can simultaneously induce a restriction on viral replication.

Discussion

A growing body of research indicates that primary infection of women by viruses such as influenza and hepatitis C virus (HCV) is characterized by an enhanced inflammatory response that can lead to viral clearance (1, 3). The present study indicates that, consistent with what has been reported in acute infection by other viruses and in vaccination studies, women exhibit a heightened cellular immune activation upon initial infection with HIV-1. The data presented shed light on how inherent sex differences in the immune response manifest in the context of HIV-1 infection, where virus is not cleared and inflammation contributes to disease progression.

While numerous studies have previously reported lower HIV-1 viral loads in chronically infected women compared to men (5-10), this study constitutes the first report wherein viral loads are compared in the absence of treatment in men and women in the same cohort, beginning in acute infection through to chronic stages. We are therefore able to demonstrate that, in this study population, women consistently have lower viral loads than men regardless of disease stage. We have further compared longitudinal $CD4^+$ T cell counts in the same population. Uninfected women in the Zambian cohort had significantly higher CD4 counts than men, as has been reported in other populations (14). As might be predicted, therefore, we observed that during very early HIV-1 infection women also have higher $CD4^+$ T cell counts than men. While this could be interpreted as reduced $CD4^+$ T cell loss in women, we demonstrate here that the rate of $CD4^+$ T cell decline in these women is actually more than two-fold greater than that in men, such that by 30 months post-EDI, there is no significant difference in the $CD4^+$ T cell counts between the two sexes. This result is consistent with a study of women enrolled in Women's Interagency HIV Study (WIHS) and men in the Multicenter AIDS Cohort Study (MACS), which also reported a faster rate of CD4 decline in women (25). However, the present

study allows for a more direct comparison of men and women by utilizing data from a single cohort where individuals have all been infected via heterosexual contact. Moreover, we show that this accelerated loss of CD4 T cells starts to occur during the first months of HIV-1 infection.

The apparent discrepancy that we and others have identified, wherein women maintain lower viral loads yet reach $CD4^+$ T cell counts indicative of extreme immunodeficiency at a similar time post-infection to men, appears to be the result of the increased cellular activation we observe in women during acute infection. Indeed, we found that total $CD4^+$ T cells in women were significantly more highly activated during acute infection, but that the difference between sexes waned over time as $CD4^+$ T cell activation increased in men. Interestingly, while the percentage of total $CD38^+$ $CD4^+$ T cells was increased in women at 1-month post-EDI versus uninfected women, it was not until the chronic stage of infection that there was a significant increase in this percentage in men. Meier et al. (11) did not observe sex differences in total $CD4^+$ T cell activation in HIV-1 infected individuals prior to ART initiation in AIDS Clinical Trials Group (ACTG) study 384, however this is consistent with our observations, since the ACTG 384 subjects were mostly chronically infected individuals with $CD4^+$ T cell counts <500 cells/mm^3. This highlights the significance of our observation that sex differences in immune activation are most prominent during acute infection, where women exhibit a more activated $CD4^+$ T cell phenotype than men.

Moreover, we found that E2 is likely a factor influencing enhanced $CD4^+$ T cell activation, due to their direct association. However, this is likely via a mechanism that involves other cells of the immune system. A significant body of research indicates that many effects of estradiol on $CD4^+$ T cells are anti-inflammatory, with evidence to suggest that estradiol inhibits

expression of the IL-2 receptor and promotes the suppressive function of regulatory CD4+ T cells (26-31). It is possible that estradiol and the estrogen receptor are involved in a positive feedback loop that contributes to the increased production of type I interferons and interferon stimulated genes (ISGs) in women, which in turn could contribute to a more generalized immune activation (11, 12, 32-35).

We also observed a striking difference in the role $CD4^+$ T cell activation plays in terms of virus replication between men and women. In men, the level of early $CD4^+$ T cell activation was significantly correlated with both contemporaneous VL and early set-point VL (determined as the geometric mean of VL between 1- and 12-months post-infection). This is consistent with a previous study of both untreated and treated MSM (15). In contrast in women no correlation between CD4+ T cell activation and VL was observed. This difference between the sexes also appears to be linked to the action of estrogen, since we observed a significant negative association between E2 levels and VL at the time of sampling; an effect that extended over the first year of infection and did not appear to be influenced by plasma levels of progesterone. Furthermore, we found that plasma levels of testosterone were in fact positively correlated with SPVL in men, although there was not a significant relationship between contemporaneous testosterone levels and viral load as was observed with E2. The strong negative correlation with E2 could provide an explanation, at least in part, for the consistently lower early and SPVL observed in women versus men.

This conclusion is supported by the observation that E2 could reduce viral replication in PBMC *in vitro*. Since cells involved in the immune response, including CD4+ T cells, are known to express the estrogen receptor, we sought to determine whether estrogen could impact the replication of authentic transmitted founder viruses in PBMC (36). While previous studies have

observed varying effects of sex hormones on HIV-1 replication, we show here that patient-derived T/F virus replication can be inhibited following treatment of PBMC with E2 (20-23). This may involve some of the same interferon-based pathways influenced by E2 that govern $CD4^+$ T cell activation, since the transcriptional levels of several ISGs have been shown to be elevated in immune cells of women and some of these may contribute to more effective control of virus replication. For example, Chang, et al. identified ISG15, which interferes with HIV-1 virion release from infected cells (37), as being more highly expressed in the $CD4^+$ T cells of women than men (12). On the other hand, Das et al. recently reported that estradiol treatment of CD4+ T cells in an *in vitro* latency model reduced reactivation of latent virus, by inducing higher levels of its receptor which bound to the HIV-1 LTR and suppressed viral transcription directly (38). Additional studies will be important to differentiate between these different possibilities. A limitation of the current study is that the data shown here regarding the relationship between E2, CD4+ T activation and viral replication are correlative, and will require further studies to identify the direct and indirect mechanisms by which these factors are related.

In this first study of sex differences in a cohort of heterosexually-infected men and women from Zambia, we have observed distinct differences in immune activation and CD4 loss during acute HIV-1 infection. We identified E2 as a significant effector molecule that can inhibit viral replication, while simultaneously exacerbating $CD4^+$ T cell activation and a more rapid loss of $CD4^+$ cells in HIV-1 infected women. Collectively, these observations suggest a complex interplay between sex hormones and cells of the immune system early in HIV infection in women, which may lead to limited control of viral load and yet exacerbate $CD4^+$ T cell death.

Materials and Methods

Study Subjects

All participants in the Zambia Emory HIV Research Project (ZEHRP) discordant couples cohort in Zambia and International AIDS Vaccine Initiative (IAVI) Protocol C were enrolled in human subjects protocols approved by both the University of Zambia Research Ethics Committee and the Emory University Institutional Review Board. Before enrollment, individuals received counseling and signed a written informed consent form agreeing to participate. All subjects selected for this study were antiretroviral therapy naïve at the time of sample collection or recording of viral load or CD4+ T cell count in accordance with standard of care and ART availability at the time (2006-2010). None of the female participants included in assays described were pregnant at the time of sample collection. The age (median and IQR) of male and female subjects is shown in Table 1.

The algorithm used to determine EDI has been previously described (13). Viral load measurements below the limit of detection (LOD) were imputed using the formula LOD/sqrt(2) (39). Set point viral load (SPVL) was calculated as the mean of $\log_{10}$ viral load values between 30 and 365 days post-EDI.

Flow Cytometry

Cryopreserved PBMCs isolated from 47 HIV-1 infected Zambians at 1, 9 and 30 months post-infection were analyzed by flow cytometry. Frozen PBMCs were thawed, washed once with

R20 [RPMI 1640 (Sigma-Aldrich, St. Louis, MO) containing 20% FBS (Sigma-Aldrich, St. Louis, MO), 1% 1 M Hepes buffer (Sigma-Aldrich, St. Louis, MO), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO), 1% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO), and 1% sodium pyruvate (Sigma-Aldrich, St. Louis, MO)]. Cells were counted using an automated cell counter (Beckman Coulter, Brea, CA) and 1 million cells were held in R10 (10% FBS) medium for staining. At that time cells were washed with 3 mL of Dulbeco's PBS without Ca/Mg (Sigma-Aldrich, St. Louis, MO) and stained for 20 min at room temperature with Aqua Live/Dead amine dye-AmCyan (Invitrogen, Carlsbad, CA). Cells were then washed once with R10 and anti-CCR7-APCR700 was added to the cells and incubated at 37° C for 15 min. The rest of the monoclonal antibodies, except anti-Ki67, were then added and incubated at room temperature for 20 min. After washing with PBS, Cytofix/Cytoperm (BD, Franklin Lakes, NJ) was added to cells and incubated at 4˚ C for 20 min. After washing with Perm wash buffer, anti-Ki67-FITC was added to the cells. The 12-parameter panel is shown in Table 2. All flow cytometry data was collected on an LSRFortessa (BD, Franklin Lakes, NJ). Analyses of these data were performed using FlowJo Version 10 software (Tree Star, Ashland OR).

Sex Steroid Hormone ELISAs

Plasma levels of 17β-estradiol, testosterone, and progesterone were measured in duplicate using commercially available ELISA assays (ThermoFisher, Waltham, MA for E2; Abcam, Cambridge, UK for testosterone and progesterone) according to the manufacturer's instructions.

Viral Replication

Transmitted/founder virus sequences for six acutely infected Zambian individuals (235234, 235028, 305136, 305156, 235227, 235212) were identified following near full-length genomic PCR amplification, and sequenced using Pacific Biosciences single molecule long read technology. Sequences were corrected for base-call errors and assembled using the MFPseq platform (40). Infectious molecular clones (IMC) were generated from second round amplicons as previously described (24). Genbank accession numbers: KR820325, KR820393, and MT347678-MT347681. Virus stocks were recovered following transfection of 293T cells as described previously (41). E2 was resuspended in 100% ethanol and stored at -20 degrees C in 100 mM aliquots. Frozen peripheral blood mononuclear cells (PBMC) from buffy coats were thawed and incubated for 72 hours in R10 (Phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum [ThermoFisher, Waltham MA] and penicillin/streptomycin) containing 20 U/ml of interleukin-2 (IL-2) and 1or 10 nM γ-irradiated 17β-estradiol or vehicle control (Sigma-Aldrich, St. Louis, MO). Cells were then infected in 24-well plates by 2 hour spinoculation at 2,200 rpm at an MOI of 0.02. After spinoculation, media in the infection plate was diluted to 2 mL total volume with the same estradiol-containing medium. On day 2 and day 3 the media was replaced with hormone-containing medium. 50μl of supernatant was sampled every 48 hours starting after replacement of cell culture media on day 3 and reverse transcriptase activity was measured at each time point using a radiolabeled reverse transcriptase assay which has been previously described (42).

Statistical Analyses

All statistical analysis was performed using GraphPad Prism, version 8 (GraphPad Software, Inc., San Diego, CA) except for generalized linear models, which were generated using JMP Pro 13 (SAS Institute, Inc., Cary, NC). Sequence analysis to determine T/F virus consensus genomes were performed with Geneious, version 9 (Biomatters, Auckland, New Zealand) using MUSCLE, followed by hand aligning. Statistical tests used are indicated in each figure legend.

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Figures and Tables

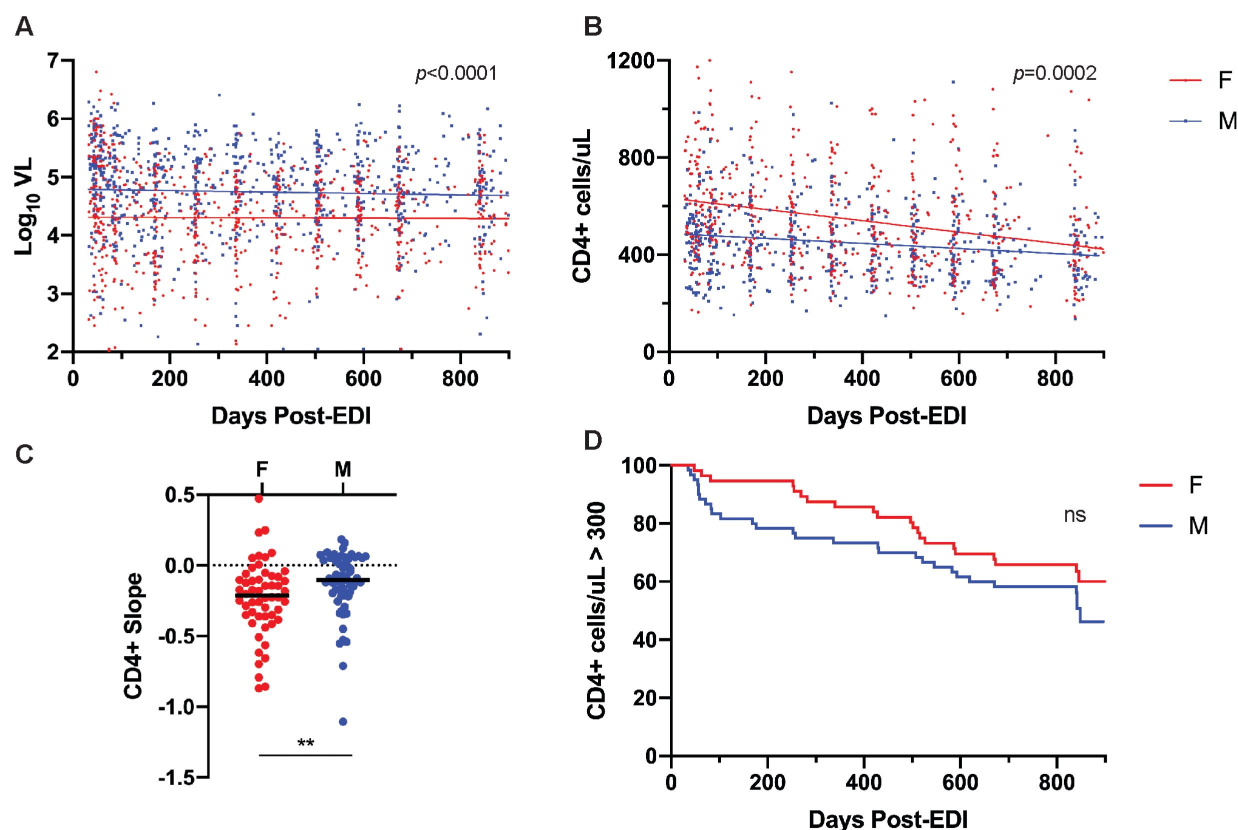

Figure 1. Faster rate of $CD4^+$ T cell decline despite lower plasma viral load in women

(A) Linear regression of $\log_{10}$ viral loads of Zambian male (n = 86) and female (n = 72) subjects between 30 and 900 days post-EDI (ANCOVA for intercepts, P < 0.0001). (B) Linear regression lines of $CD4^+$ T cells counts from male (n = 60) and female (n = 56) subjects between 30 and 900 days post EDI (ANCOVA for slopes, P = 0.0002). (C) Comparison of slopes of individual linear regression lines of $CD4^+$ cell counts between 30 and 900 days post-EDI for individuals in Fig. 1(B), (Mann-Whitney U test, P = 0.005). (D) Survival curves indicating time to $CD4^+$ cell count < 300 cells/μL by sex (Log-rank test, P = 0.2).

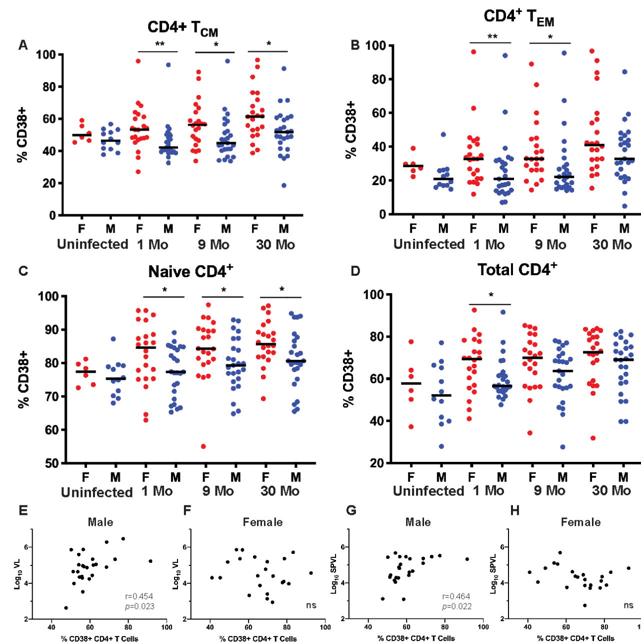

Figure 2. $CD4^+$ T cells in women are more highly activated than men but do not correlate with SPVL

Proportion of $CD4^+$ T cells expressing CD38 in uninfected women and men, and individuals at 1, 9 and 30 months post-EDI in (A) T_{CM} cells ($CCR7^+CD27^+CD45RO^+$) (Mann-Whitney U test, P = 0.004, P = 0.037, and 0.010 at 1, 9 and 30 months post-EDI, respectively) (B) T_{EM} cells ($CCR7^-CD27^-CD45RO^+$) (Mann Whitney U test, P = 0.009 and P = 0.013, at 1 and 9 months post-EDI respectively) (C) T_{Naive} cells ($CCR7^+CD27^+CD45RO^-$) (Mann-Whitney U test, P = 0.032, P = 0.025, and P = 0.023 at 1, 9 and 30 months post-EDI, respectively) and (D) Total $CD4^+$ T cells (Mann Whitney U test, P = 0.024 1 month post-EDI). (E) Correlation between proportion of $CD4^+$ T cells expressing CD38 at 1-month post-EDI and $Log_{10}VL$ at time of sampling in men (Spearman's correlation, P = 0.023 r = 0.454) and (F) women (Spearman's correlation, P = 0.471 r = -0.162) (G) Correlation between proportion of $CD4^+$ T cells expressing CD38 at 1-month post-EDI and SPVL in men (Spearman's correlation, P = 0.022 r = 0.464) and (H) women (Spearman's correlation, P = 0.101 r = -0.359).

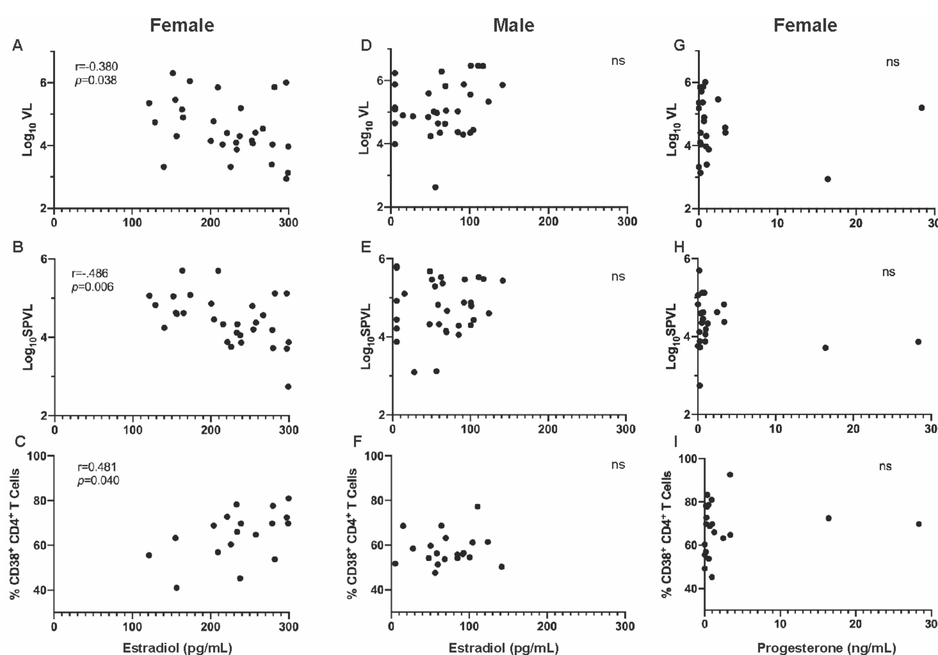

Figure 3. Plasma levels of 17β-estradiol but not progesterone at 1-month post infection correlate with VL, SPVL, and CD4$^+$ T cell activation in women

17β-estradiol (E2) was measured in the plasma at 1 month post-infection. (A) Correlation between plasma levels of E2 and Log_{10} VL at 1 month post-EDI in women (Spearman's correlation, P = 0.038, r = -0.380) and (D) men (Spearman's correlation, P = 0.241, r = -0.184). (B) Correlation between plasma levels of E2 and Log_{10} SPVL in women (Spearman's correlation, P = 0.006, r = -0.486) and (E) men (Spearman's correlation, P = 0.782, r = -0.051). (C) Correlation between plasma levels of E2 and CD4$^+$ T cell activation at 1 month post-EDI in women (Spearman's correlation, P = 0.0404, r = 0.487) and (F) men (Spearman's correlation, P = 0.582, r = 0.131). (G) Correlation between plasma levels of progesterone and Log_{10} VL, (H) Log_{10} SPVL, and (I) CD4$^+$ T cell activation at 1 month post-EDI in women (Spearman's correlation, P = 0.640, r = -0.12; P = 0.527, r = -0.14 and; P = 0.329, r = 0.224, respectively).

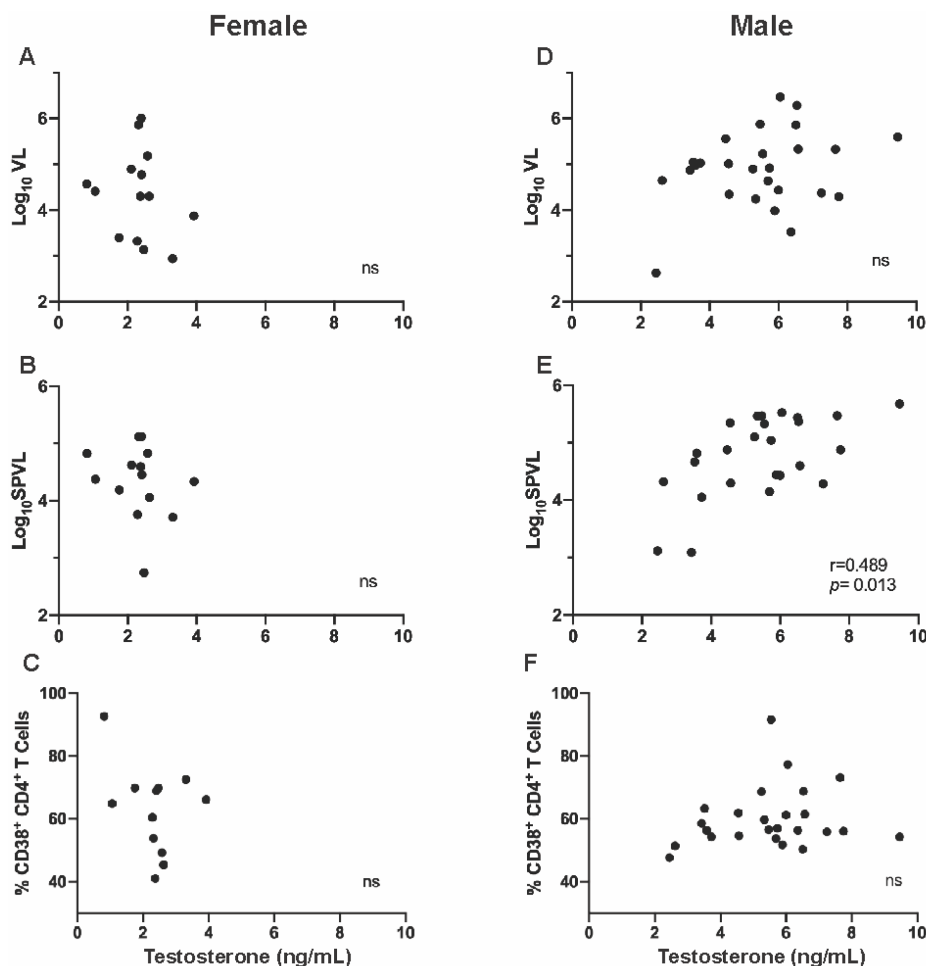

Figure 4. Relationship between plasma levels testosterone at 1-month post infection and VL, SPVL, and $CD4^+$ T cell activation.

Testosterone was measured in the plasma at 1 month post-infection. (A) Correlation between plasma levels of testosterone and Log_{10} VL at 1 month post-EDI in women (Spearman's correlation, P = 0.445, r = -0.22) and (D) men (Spearman's correlation, P = 0.304, r = 0.210). (B) Correlation between plasma levels of testosterone and Log_{10} SPVL in women (Spearman's correlation, P = 0.292, r = -0.30) and (E) men (Spearman's correlation, P = 0.013, r = 0.489). (C) Correlation between plasma levels of testosterone and $CD4^+$ T cell activation at 1 month post-EDI in women (Spearman's correlation, P = 0.592, r = -0.17) and (F) men (Spearman's correlation, P = 0.495, r = 0.143)

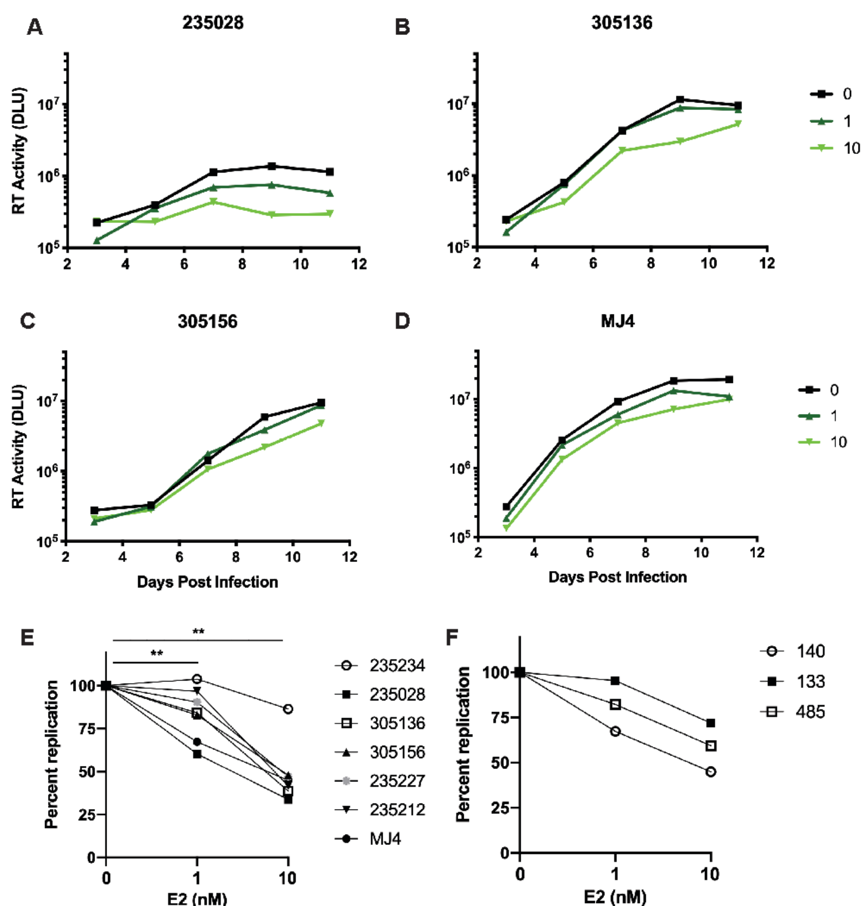

Figure 5. Estradiol inhibits replication of transmitted/founder viruses and MJ4

Transmitted/founder viruses as well as MJ4 were used to infect PBMC from a healthy female donor (140) in media containing 0, 1, or 10 nM E2. (A-D) Representative *in vitro* viral replication curves over an eleven-day period for T/F viruses from 235028, 305136, 305156, and MJ4. The median value of 3 technical replicates is shown for each treatment at each time point. (E) Percent replication in treated compared to untreated control cells as determined from AUC in six T/F viruses and MJ4 between days 3 and 11 for each of the treatment groups (Freidman test with Dunn's multiple comparisons tests, P = 0.003 between 0 and 10nM E2, P = 0.008 between 0 and 1 nM). (F) Percent replication in treated compared to untreated control cells as determined from AUC of MJ4 between days 3 and 11 in Donors 140, 133, and 485.

	Female	Male
Fig. 1A	32 (26-36.5), n = 72	35 (31-41), n = 86
Fig. 1B	31.5 (25.25-35.75), n = 56	34 (30.25-39), n = 60
Fig. 2	30 (25-33), n = 22	33 (28.5-37.5), n = 25

Table 1. Age (IQR) for male and female subjects.

Antibody target	**Clone**	**Fluorochrome**	**Manufacturer**
Viability	N/A	Amcyan	Invitrogen
CD3	SK7	APC H7	BD Pharmingen
CD4	RPA-T4	BV 605	Biolegend
CD8	RPA-T8	BV655	BD Horizon
CD45RO	UCLH1	PECF594	BD Horizon
CCR7	3D12	APCR700	BD Horizon
CD27	M-T271	PeCy7	BD Pharmingen
HLADR	L243	PE	EBioscience
CD38	HB7	APC	BD Horizon
PD1	EH12.1	BV421	BD Horizon
Ki67	35/Ki67	FITC	BD Horizon
CD57	HNK-1	PerCP Cy5.5	Biolegend

Table 2. Flow cytometry panel for immunophenotyping.

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Chapter III: Heightened expression of type I interferon signaling genes in CD4+ T cells from acutely HIV-1 infected women contributes to lower viral loads

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Abstract

Sex differences play a role in the pathogenesis of a number of viral diseases. In HIV-1, several studies have reported that chronically infected women have significantly lower plasma viremia than men, although the exact mechanism by which this occurs has yet to be identified. We have performed bulk RNA-seq experiments comparing gene expression between $CD4^+$ T cells from acutely HIV-1 infected men and women in Zambia, since we observe lower viral load (VL) despite higher CD4 activation in these women during acute/early infection. In a univariate analysis we have identified a number of differentially expressed genes. However, after controlling for differences in VL and $CD4^+$ cell count between men and women we identify a large set of genes more highly expressed in women that are significantly enriched for type I interferon (IFN) signaling genes. Genes more highly expressed in men show no such enrichment. We then identify a subset of these genes, including several involved in type I IFN signaling (*IRF7*, *DDX58*, *SAMHD1*, *OAS2*, and *TRIM14*), that are both more highly expressed in $CD4^+$ T cells from women and negatively correlated with VL, indicating that they may play a role in the comparative control of VL observed in women.

Introduction

Sex differences in pathogenesis of a number of viral diseases have been well documented in the literature (1). In HIV-1, several studies have reported that chronically infected women have significantly lower plasma viremia than men (2-7). We have recently shown that this discrepancy in viral load (VL) begins during acute infection and remains consistent throughout the duration of infection (8). However, the exact mechanism by which women maintain consistently lower VL than men, despite more activated $CD4^+$ T cells early in infection, has yet to be elucidated. Here we describe gene expression analysis of $CD4^+$ T cells from acutely HIV-1 infected men and women to identify genes that may contribute to this phenomenon.

During HIV-1 infection, type I interferons (IFNs) engage in a number of antiviral activities including degradation of RNA, arrest of cell cycle progression, promotion of antigen presentation, and induction of apoptosis in infected cells while simultaneously contributing to the induction of chronic immune activation (9, 10). This is evident in the rhesus macaque model, where IFNα administration was shown to initially upregulate expression of IFN-stimulated genes (ISGs) and prevent systemic SIV infection. However, continued IFNα treatment also resulted in accelerated $CD4^+$ T cell depletion and increased viremia (11). In agreement with the results obtained from the primate models of SIV infection, several studies have reported a strong activation of different components associated with the type I IFN response (including IRFs, ISGs and viral DNA sensors) during chronic HIV-1 infection (12-16). High levels of ISGs such as IP-10 are also associated with more rapid $CD4^+$ T cell depletion (17, 18).

Previous gene expression analysis demonstrated clearly that expression of ISGs increases in HIV-infected individuals in various cell types (12, 13, 19, 20). However, a study of chronically HIV-1 infected individuals found that several ISGs were expressed at significantly

higher levels by $CD4^+$ T cells (as well as $CD8^+$ T cells and plasmacytoid dendritic cells [pDCs]) from women than men, but only after correcting for differences in VL (21). It has additionally been demonstrated that pDCs from women produce significantly higher amounts of IFNα than those from men after stimulation with HIV-1 antigens, implying a more activated phenotype given similar antigenic stimulation (22). It is therefore conceivable that both female sex and antigenic stimulation with HIV-1 contribute to expression of ISGs that play a role in the immune response to HIV-1 infection.

Much of the work describing sex differences during viral infection focuses in pDC, the primary producers of IFN alpha (IFNα). Comparatively little is known about sex differences in gene expression between $CD4^+$ T cells, the primary target cell of HIV-1. Additionally, previous studies of sex differences in HIV-1 pathogenesis have focused on the chronic phase of infection or in antiretroviral therapy (ART)-treated individuals. Here we describe experiments comparing gene expression between $CD4^+$ T cells from acutely HIV-1 infected men and women in Zambia who were ART naïve at the time of sample collection, allowing for comparison of natural infection in the absence of treatment. We identify a subset of genes, including several involved in type I IFN signaling, that are both more highly expressed in $CD4^+$ T cells from women and negatively associated with VL, indicating that they may play a role in the comparative control of VL observed in women.

Results

Type I IFN-related genes are differentially expressed between $CD4^+$ T cells of acutely HIV-1 infected men and women

We performed bulk RNA-seq on FACS-sorted $CD4^+$ T cells from cryopreserved PBMC of 4 men and 4 women acutely infected with HIV-1 (approximately 3 months post-EDI). All subjects were enrolled in the International AIDS Vaccine Initiative (IAVI) Protocol C in Lusaka, Zambia and were ART naïve at the time of sampling, in compliance with protocols at the time of sample collection (2006-2009). Additional subject demographic information is listed in Table 1.

Live $CD4^+$ T cells were sorted into effector memory (T_{EM}, $CCR7^-CD45RA^+$), central memory (T_{CM}, $CCR7^+CD45RA^+$), and naïve populations (T_{naive}, $CCR7^+CD45RA^-$). Data generated by combining read counts from these 3 populations are referred to as total $CD4^+$ T cells. We found in total $CD4^+$ T cells that 168 genes were more highly expressed in women than men (Fig. 1A). While we do not find statistically significant enrichment for immune system pathways, several genes in the type I IFN signaling pathway were expressed at significantly higher levels in women than men. These were: *IRF7*, *USP18*, *ISG15*, *IFI27*, and *IFI6* (Fig. 1B). IRF7 is a master regulator of type I IFN production downstream of viral sensors (23). *ISG15* encodes an E3 ubiquitin ligase with anti-HIV activity and *USP18* encodes is an ISG15-specific protease, serving as a counterweight to ISG15 function (24).

In men, 308 genes were expressed by total $CD4^+$ T cells to a greater extent than in women. In this gene set we find modest enrichment for genes associated with both positive and negative regulation of T cell activation as well as positive and negative regulation of apoptotic

processes. In order to de-convolute the effect of sex and viral stimulation on expression of these genes, we next performed a multivariate analysis controlling for $CD4^+$ cell count and VL.

Immune system signaling pathways are significantly enriched in genes more highly expressed by $CD4^+$ T cells from women after controlling for VL and $CD4^+$ cell count

Previous studies have demonstrated that healthy as well as acutely HIV-1 infected women have significantly higher numbers of $CD4^+$ T cells than men (8, 25). Women have additionally been demonstrated to have consistently lower VL than men throughout HIV-1 infection (2-8). Therefore, in order to understand how sex affects $CD4^+$ T cell gene expression in the absence of these differences in viral stimulation and target cell number, we performed a multivariate regression analysis examining differential expression of genes while controlling for $CD4^+$ T cell count and VL of each individual at the time of sampling.

In this analysis, we found that 710 genes were more highly expressed in women than men (Fig. 2A). In this gene set, we found significant enrichment for genes involved in the response to type I IFN (GO: 0060337, $p_{adj} = 7.95x10^{-6}$) (Fig. 2B). These include a number of genes that encode proteins with direct antiviral function including *IFITM1*, *SAMHD1*, *MX1*, and *OAS1-3*. Importantly, the proteins encoded by *IFITM1* and *SAMHD1* have direct anti-HIV function, interfering with virus entry and replication, respectively (26-28). Other genes that meet this criteria code for proteins that are directly involved with type I IFN signal transduction including: *IFNAR1*, *IRF7*, and *STAT1*. 1182 genes were more highly expressed by $CD4^+$ T cells from men than women, however in this gene set we found no enrichment for pathways involved in immune system function.

Expression of 2097 genes are significantly associated with VL

In order to understand how gene expression contributes to differential VL observed in men and women, we first performed a univariate analysis of VL versus gene expression. Using a generalized linear model framework, we analyzed differential expression of genes per unit change in VL. Using this model, we found differential expression of 2097 whose expression was significantly correlated with VL. 909 of these were positively correlated with VL (positive log_2fold change per unit increase in VL). Selected examples of these correlations are shown in Figure 3. *CXCR4*, which encodes one of the two primary co-receptors for HIV-1 was positively correlated with VL (Fig 3A). Additionally, a number of genes in the type I IFN signaling pathway are also positively correlated with VL including *IRF1* (Fig 3B). This is in agreement with numerous studies, as interferon expression is stimulated by viral sensing (12, 13, 19, 20). Furthermore, expression of a number of genes encoding T cell activation molecules are positively associated with VL including *CD28* and *CD69*, consistent with the fact that HIV-1 induces significant T cell activation (Fig 3C) (29).

We additionally found that expression of 1188 genes was negatively associated with viral load (negative log_2fold change per unit increase in viral load). A number of members of the TRIM family of E3 ubiquitin ligases including *TRIM2*, *TRIM14*, *TRIM27*, *TRIM46*, *TRIM58*, and *TRIM62* (Fig. 3D), were all negatively associated with VL. Furthermore, we found that genes associated with cell death including *CASP10* (Fig. 3E) and *CASP1* were negatively associated with VL. Finally, expression of inhibitory receptors including *LAIR1* (Fig 3F), *CISH*, and *SOCS2* were also negatively associated with VL.

51 genes more highly expressed in women are also associated with lower viral load

In order to understand how genes expressed to a greater extent in women can contribute to lower viral loads we determined which genes were expressed more highly in women and also were associated with lower VL. 51 genes met these criteria. The full list of differentially expressed genes that are both more highly expressed in women and negatively associated with viral load can be found in Table S1. This gene set contains significant enrichment for immune system processes (GO:0002376, p_{adj}= 0.032) (Fig. 4A). These include cell death pathways (*CASP1*, *TNFSF10*, *BCL2L1*) as well as two signaling lymphocytic activation maker (SLAM) family members: *SLAMF6* and *LY9*. It also includes two genes that encode antiviral E3 ubiquitin ligases, *DTX3L* and *TRIM14*, the latter in particular has been reported to be an IFN-stimulated HIV-1 restriction factor (30). Several other genes in this set code for genes that are involved in various stages of type I IFN signaling. *DDX58* encodes the viral RNA sensor RIG-I, which triggers downstream IFN signaling cascades (Fig. 4B) (31). *IRF7* is a master regulator of type I IFN production critical for the antiviral response (Fig. 4C) (23). As described above, *SAMHD1* and *OAS2* are IFN-stimulated genes with direct antiviral function (Fig. 4D and E).

Discussion

We have demonstrated here that a significant number of genes involved in important immune system functions are differentially expressed in the CD4$^+$ T cells of women compared to men during acute/early HIV-1 infection. Many of these differences in expression can be observed without controlling for the known differences in HIV-1 VL and CD4$^+$ T cells that exist between men and women. However, by controlling for these factors, are able to identify a large number of genes and identify specific functional pathways that may directly contribute to control of VL commonly observed in HIV-1 infected women.

Without controlling for VL and CD4$^+$ cell count, we have identified 476 genes that are differentially expressed between CD4$^+$ T cells of acutely HIV-1 infected men and women. Several genes involved in the type I IFN signaling pathway were expressed at significantly higher levels in women than men. This is consistent with previous reports that ISGs were more highly expressed from CD4$^+$ T cells (in addition to CD8$^+$ T cells and pDC) in chronically infected women than men (21). Specifically, we confirm that ISG15, a known HIV-1 restriction factor, is more highly expressed in CD4$^+$ T cells in women. Importantly, we show here that these differences in expression are present from early in infection and may therefore set the stage for later differences in pathogenesis. We also identify heightened expression of additional IFN signaling genes *IRF7*, *USP18*, *IFI27*, and *IFI6*. This demonstrates heightened expression in women of genes throughout the type I IFN signaling pathway including a master regulator of type I IFN production (*IRF7*) critical for the antiviral response (23).

When controlling for differences in VL and CD4$^+$ cell count between men and women, the differences in gene expression are even more apparent. We find significant enrichment for a number of immune signaling pathways among genes more highly expressed in women,

particularly those involved in the response to and production of type I IFN. In this multivariate regression model, we identified 710 genes were more highly expressed by $CD4^+$ T cell from women than men. In this gene set, we found significant enrichment for genes involved in the response to type I IFN, including a number of genes that code for proteins with direct antiviral function: *USP18*, *IFITM1*, *SAMHD1*, *MX1*, and *OAS1-3*. Others are directly involved with IFN signal transduction including: *IFNAR1*, *IRF7*, and *STAT1*. While an even greater number of genes were more highly expressed by $CD4^+$ T cells from men, we found no enrichment for pathways involved in immune system function, highlighting that this heightened type I IFN response to acute infection is specific to women.

Importantly, we have also identified a large number of genes whose expression was directly correlated with VL regardless of patient sex. Many of these are validated by previous work. For example, expression of ISGs are known to increase in HIV-infected individuals in various cell types (12, 13, 19, 20). As would be expected, we find that some ISGs, including *IRF1* and *IRF8* are positively associated with VL. Furthermore, a number of T cell activation molecules are positively associated with VL including CD28 and CD69.

However, we also identified a number of ISGs whose expression negatively correlates with VL, including *IRF7* and *IRF3*. Type I IFNs engage in a number of antiviral activities, however they simultaneously contribute to the induction of chronic immune activation (10). This is evident in the rhesus macaque model, where IFNα administration was shown to initially upregulate expression of IFN-stimulated genes (ISGs) and prevent systemic SIV infection but continued IFNα treatment also resulted in accelerated $CD4^+$ T cell depletion and increased viremia (11). It is possible that the ISGs negatively correlated with VL are those that

impart sufficient restriction on viral replication, although additional experimentation *in vitro* will be required to discern the causality of these relationships.

Nevertheless, many of the identified genes have no known relationship to HIV-1 pathogenesis. For example, we identified a number of TRIM E3 ubiquitin ligases (*TRIM2*, *TRIM27*, *TRIM46*, *TRIM58*, *TRIM62*) whose expression negatively correlated with VL that have not been previously implicated in restricting HIV-1 replication. Further analyses and experimentation are likely to reveal additional potential regulators of HIV-1 pathogenesis.

Finally, we demonstrated that a number of genes which are more highly expressed by $CD4^+$ T cells in women than men during acute infection were also significantly correlated with lower VL. This gene set is significantly enriched in genes involved in the response to type I IFN including *IRF7*, *DDX58*, *OAS2*, *TRIM14*, and the HIV-1 restriction factor *SAMHD1*. While $CD4^+$ T cells are not the primary producers of type I IFN, it is possible that the effects of differential type I IFN production by pDCs between the sexes results in the disparate gene expression that we observe here (32).

CASP1, which encodes the pro-inflammatory Caspase-1, was additionally more highly expressed by women and inversely correlated with VL. Our previous data demonstrates that women lose $CD4^+$ cells during HIV-1 infection at over twice the rate of men (8). Identification of *CASP1*, which mediates cell death through pyroptosis, in this group of genes suggests that this death mechanism may play a role in the increased rate of $CD4^+$ cell loss in women. While the exact mechanisms of $CD4^+$ T cell loss during HIV-1 infection have not been fully elucidated, recent reports suggest that the Caspase-1 mediated pyroptosis provides the main mechanism (33). It remains to be seen whether other markers of pyroptosis are also increased in $CD4^+$ T cells from HIV-1 infected women compared to men, particularly at the protein level.

As a whole, these data help to elucidate important differences in gene expression during the acute phase of HIV-1 infection and identify several antiviral and pro-inflammatory genes that may contribute to the consistent control of viral replication observed in HIV-1 infected women.

Materials and Methods

Study Subjects

All participants in the Zambia Emory HIV Research Project (ZEHRP) discordant couples cohort in Zambia and International AIDS Vaccine Initiative (IAVI) Protocol C were enrolled in human subjects protocols approved by both the University of Zambia Research Ethics Committee and the Emory University Institutional Review Board. Before enrollment, individuals received counseling and signed a written informed consent form agreeing to participate. All subjects selected for this study were antiretroviral therapy naïve at the time of sample collection or recording of viral load or CD4+ T cell count in accordance with standard of care and ART availability at the time (2006-2009). None of the female participants included in assays described were pregnant at the time of sample collection. The algorithm used to determine EDI has been previously described (34). SPVL was calculated as the mean of log_{10} viral load values between 30 and 365 days post-EDI.

Cell Sorting and RNA-seq

Cells were sorted on a FACS Aria III (BD, Franklin Lakes, NJ). Cells were sorted into $CD4^+$ T_{naive} ($CCR7^+CD45RA^+$), T_{EM} ($CCR7^-CD45RA^-$), and T_{CM} ($CCR7^+CD45RA^-$) subsets. Total $CD4^+$ T cells refers to data combining normalized gene expression from these subsets. An average of 15,000 cells were sorted at 4° C into RNAse-free eppendorf tubes containing RPMI. Cells were then centrifuged at 600 x g for 10 minutes and supernatant was removed. 350uL RLT buffer (Qiagen, Hilden, Germany) plus 1% BME was added and cells were vortexed for 1 min. Lysed cells were then stored at -80° C and submitted to the Yerkes NHP Genomics Core for

RNA extraction (RNeasy Micro, Qiagen, Hilden, Germany), cDNA synthesis (SMART-seq v4, Takara, Kusatu, Japan) and sequencing on a HiSeq3000 (Illumina, San Diego, CA) at a depth of 17-20 million reads per sample at 100SR.

Data Analysis

Sequencing reads were aligned to a reference genome GRCh38 using STAR (35). Differential expression analysis was then performed using DESeq2, including internal normalization to correct for library size and RNA composition bias and shrinkage estimation for dispersions and fold changes (36). All statistical tests were corrected for multiple testing to obtain adjusted p-values (p_{adj}). Genes with p values less than 0.05 and adjusted p-values less than 0.20 are deemed DE. Volcano plots were generated using EnhancedVolcano (37) in R Studio Version 1.2 (Boston, MA).

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Figures and Tables

Subject ID	Sex	Age	Days Post-EDI	$Log_{10}VL$	$Log_{10}SPVL$	CD4 Count (cells/mm^3)
235095	F	22	49	5.74	5.44	475
305123	M	29	82	5.57	5.62	284
235072	M	26	87	5.16	5.47	335
235216	M	29	86	4.52	4.43	515
235092	M	27	112	4.20	4.30	527
235074	F	25	89	4.07	4.60	525
235086	F	23	84	3.21	3.71	738
235036	F	35	138	3.00	5.00	545

Table 1. Subject information

Demographic and sample information from 8 subjects included in RNA-seq experiments.

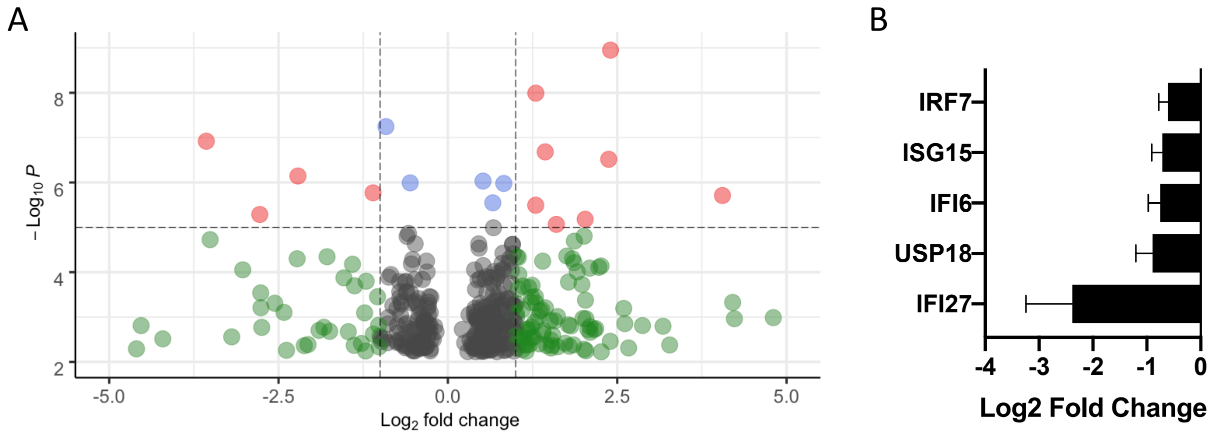

Figure 1. Differential expression of genes between CD4+ T cells of acutely HIV-1 infected men and women.

(A) Differential expression of genes in women compared to men displayed as $\log_2$ fold change (male – female) (FDR<0.20). Genes on X and Y chromosomes are not shown ($-\text{Log}_{10}$P values are beyond the Y axis) (B) Type I interferon signaling genes more highly expressed in women.

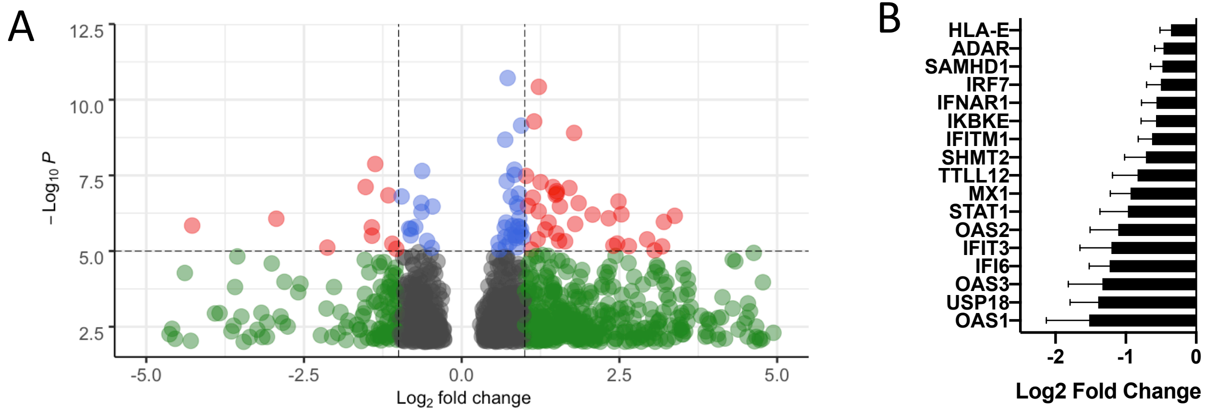

Figure 2. Differential expression of genes between men and women when controlling for SPVL and CD4+ T cell count.

(A) Differential expression of genes in women compared to men displayed as $\log_2$ fold change (male – female) (FDR<0.20). (B) Differential expression of type I IFN responsive genes in women (GO: 0060337, $p_{adj} = 7.95\text{x}10^{-6}$).

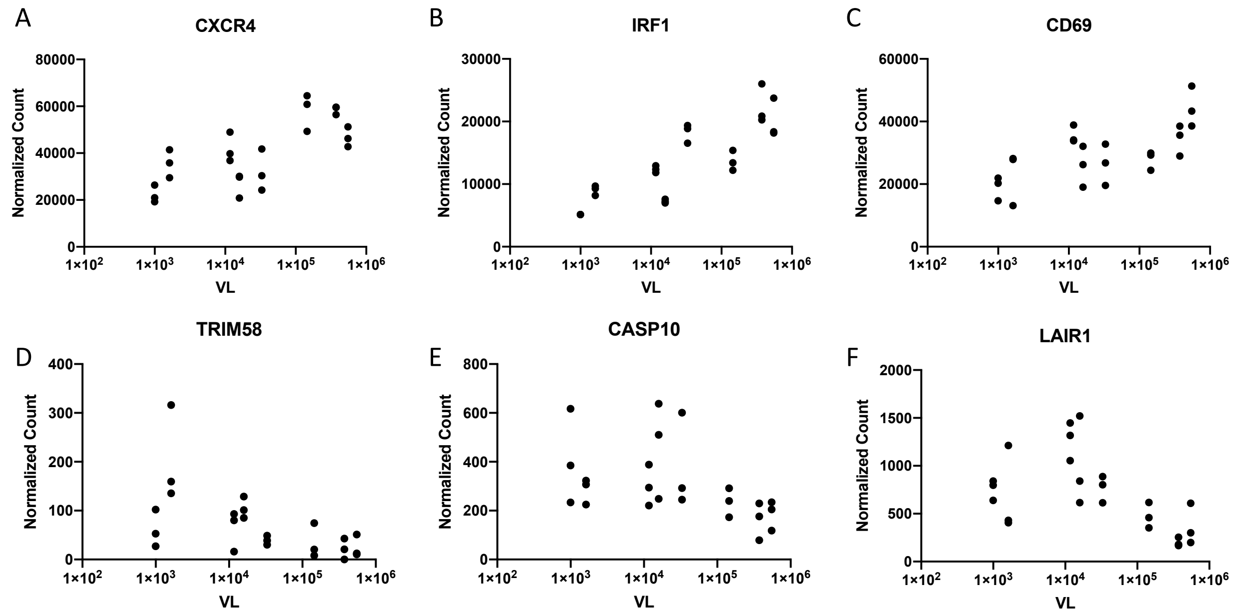

Figure 3. Expression of selected genes correlated with VL.

Normalized expression of (A) CXCR4 (p_{adj} = 0.036), B) IRF1 (p_{adj} = 0.004), (C) CD69 (p_{adj} = 0.024), (D) TRIM58 (p_{adj} = 0.034) (E) CASP10 (p_{adj} = 0.016), and (F) LAIR1 (p_{adj} = 0.002) versus subject viral load. Data points with identical VL represent normalized gene expression $CD4^+$ T_{naive} T_{EM}, T_{CM} subsets from a single subject.

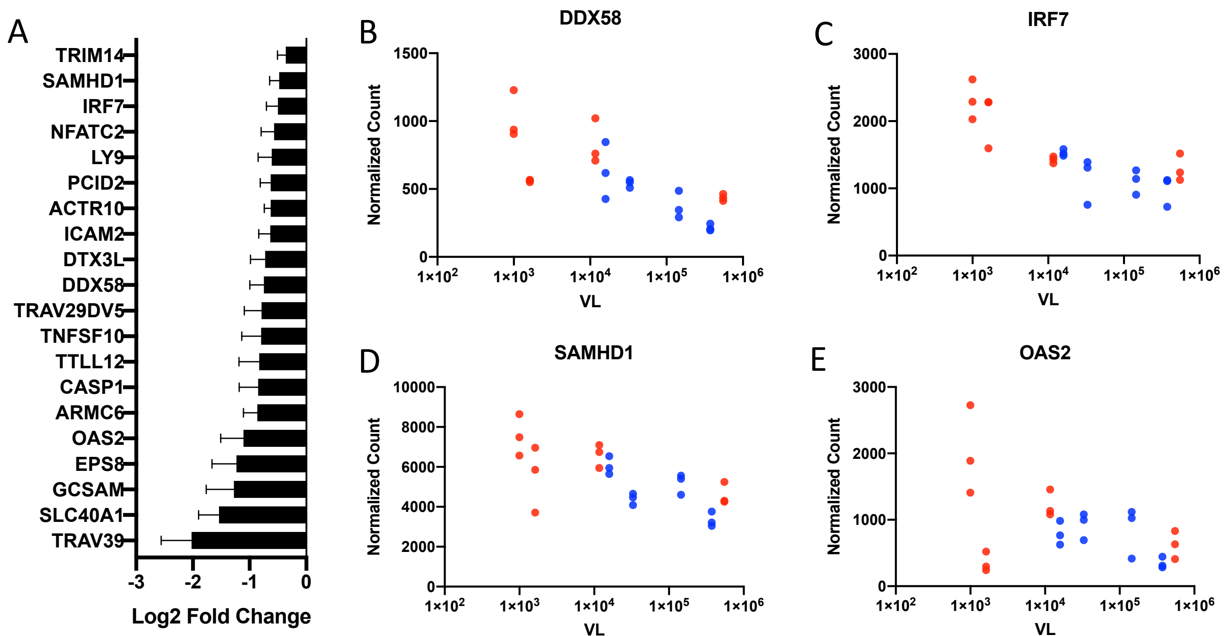

Figure 4. 51 genes more highly expressed in women are also associated with lower viral load

(A) Genes that are both more highly expressed in CD4+ T cells from women and inversely correlated with viral load and fall under the GO term GO:0002376, Immune System Process. Representative graphs of these genes involved in type I interferon signaling (B) DDX58, (C) IRF7, (D) SAMHD1, and (E) OAS2. Data from female subjects is shown in red and male subjects in blue. Data points with identical VL represent normalized gene expression $CD4^{+}$ T_{naive} T_{EM}, T_{CM} subsets from a single subject.

ENSEMBL ID	Gene Name	Chromosome
ENSG00000250182	EEF1A1P13	5
ENSG00000166578	IQCD	12
ENSG00000173262	SLC2A14	12
ENSG00000158748	HTR6	1
ENSG00000171747	LGALS4	19
ENSG00000258405	ZNF578	19
ENSG00000211818	TRAV39	14
ENSG00000138449	SLC40A1	2
ENSG00000120833	SOCS2	12
ENSG00000167083	GNGT2	17
ENSG00000174500	GCSAM	3
ENSG00000151491	EPS8	12
ENSG00000114737	CISH	3
ENSG00000111335	OAS2	12
ENSG00000235173	HGH1	8
ENSG00000162739	SLAMF6	1
ENSG00000171552	BCL2L1	20
ENSG00000196636	SDHAF3	7
ENSG00000105676	ARMC6	19
ENSG00000175567	UCP2	11
ENSG00000137752	CASP1	11
ENSG00000100304	TTLL12	22
ENSG00000215114	UBXN2B	8
ENSG00000121858	TNFSF10	3
ENSG00000211810	TRAV29DV5	14
ENSG00000133574	GIMAP4	7
ENSG00000107201	DDX58	9
ENSG00000100246	DNAL4	22
ENSG00000163840	DTX3L	3
ENSG00000129925	PGAP6	16
ENSG00000137168	PPIL1	6
ENSG00000080845	DLGAP4	20
ENSG00000164961	WASHC5	8
ENSG00000108622	ICAM2	17
ENSG00000131966	ACTR10	14
ENSG00000126226	PCID2	13
ENSG00000122224	LY9	1
ENSG00000144560	VGLL4	3
ENSG00000016864	GLT8D1	3
ENSG00000101096	NFATC2	20
ENSG00000204261	PSMB8-AS1	6
ENSG00000163947	ARHGEF3	3
ENSG00000185507	IRF7	11
ENSG00000101347	SAMHD1	20
ENSG00000181924	COA4	11
ENSG00000127483	HP1BP3	1
ENSG00000196591	HDAC2	6
ENSG00000106785	TRIM14	9
ENSG00000183431	SF3A3	1
ENSG00000144744	UBA3	3
ENSG00000090273	NUDC	1

Table S1. Genes more highly expressed in CD4+ T cells than women and negatively associated with viral load.

Cell Marker	mAb Clone	mAb Fluorophore	Manufacturer
CD3	SP34-2	Alexa 700	BD
CD4	L200	PE	BD
CD8α	RPA-T8	BV421	Biolegend
CD45RA	HI100	FITC	Biolegend
CCR7	3D12	PE-Cy7	BD

Table S2. Antibody panel for cell sorting

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Chapter IV: Discussion

Significance

We have described herein studies that demonstrate the significant sex differences that exist in the pathogenesis of HIV-1 infection in a cohort of Zambian men and women. Our primary epidemiological observation that women have lower HIV-1 viral load (VL) than men is in agreement with many years of data from cross-sectional cohorts (1-5). However, our focus on acute infection is of substantial importance. We have demonstrated in this work that even at the earliest time after infection women establish a consistently lower plasma VL than men, and that this is maintained into early chronic infection. This observation forms the basis for the remainder of our studies which focus on the acute stage of HIV-1 infection and how early events can set the stage for future disease progression.

Though our collaboration with the Zambia Emory HIV Research Project (ZEHRP) we were able to utilize data spanning 30 months of infection in the absence of antiretroviral therapy (ART). Thus, we were able to calculate the rate of $CD4^+$ decline for over 50 individuals of each sex and identify a more than 2-fold higher rate of $CD4^+$ cell loss in women than men. This is significant because data from some chronic infection cohorts has suggested there is no difference in the time required for HIV-1 infected men and women meet clinical endpoints or progress to AIDS (<200 $CD4^+$ T cells/mm^3) (1). Without understanding the full trajectory of the disease beginning in acute infection, one could interpret this data as implying there is not difference in disease course between men and women. Instead, we demonstrate here that although healthy women have more $CD4^+$ T cells than men, $CD4^+$ T cell activation early in HIV-1 infection contributes to the increased rate of $CD4^+$ T cell loss in women (6).

We observed that women had significantly higher percentages of $CD38^+CD4^+$ T cells during acute infection and that this difference waned over time. The percentage of $CD38^+CD4^+$ T cells was higher in women in effector and central memory $CD4^+$ T cells as well as naïve cells. Interestingly, the loss of significant sex difference over time appeared to be the result of a gradual increase in the percentage of $CD38^+CD4^+$ T cells in men, so that the percentage of activated $CD4^+$ T cells were approximately equal by 9 to 30 months post-EDI. Importantly, the percentage of $CD38^+CD4^+$ T cells during acute infection in men correlated with VL, as would be expected given that activated $CD4^+$ T cells are the preferred target for replicating HIV-1 virus (7). In women, we were not able to observe such a relationship, implying that the dynamics of virus production from activated $CD4^+$ T cells may differ between men and women. We consider these early events to be of significant interest as they may provide insight into mechanisms of $CD4^+$ T cell loss during HIV-1 infection in general.

We therefore examined the gene expression profiles of $CD4^+$ T cells isolated from acutely infected men and women. We demonstrated that a number of genes are more highly expressed by $CD4^+$ T cells in women than men during acute infection. Interestingly, the identification of CASP1 (which encodes the pro-inflammatory caspase Caspase-1) in this gene set suggests that pyroptosis may play a role in the increased rate of $CD4^+$ T cell loss in women. While the exact mechanisms of $CD4^+$ T cell loss during HIV-1 infection remains a matter of debate, recent reports suggest that the Caspase-1 mediated pyroptosis provides the main mechanism (8). It remains to be seen whether this and other markers of pyroptosis are also increased in $CD4^+$ T cells from HIV-1 infected women at the protein level.

We further examined the role of sex hormones, and estradiol (E2) in particular, in modulation of $CD4^+$ T cell activation and HIV-1 replication. We found that in women, activation

of $CD4^+$ T cells in the acute phase of infection was significantly correlated to plasma levels of E2. These levels in early infection were also associated with lower early and set-point VL (SPVL) in women, and were able to significantly restrict HIV-1 replication *in vitro*. However, additional experimentation is needed to discern the exact mechanisms by which E2 affects these parameters.

The mechanism by which E2 relates to $CD4^+$ T cell activation likely involved other cells of the immune system. Research focused on the higher levels of E2 observed during pregnancy indicates that it can induce effects on $CD4^+$ T cells are anti-inflammatory, with evidence to suggest that estradiol inhibits expression of the IL-2 receptor and promotes the suppressive function of regulatory $CD4^+$ T cells (9-14). However, it is possible that E2 and the estrogen receptor are involved in a positive feedback loop that contributes to the increased production of type I interferon (IFN) and IFN-stimulated genes (ISGs) in women, which in turn could contribute to a more generalized immune activation (15-20). In fact, we have identified here a number of IFN-responsive genes that are more highly expressed in the $CD4^+$ T cells of acutely infected women compared to men. In our experiments, we find that IRF7, USP18, ISG15, IFI27, and IFI6 are more highly expressed in women. While we have not examined pDC function in this cohort, it is likely that the increased expression of these genes in $CD4^+$ T cells results from increased production of type I IFNs by pDCs in women, as has been observed previously (15, 21).

Importantly, we also observed a strong inverse correlation between plasma levels of E2 during acute infection and both contemporaneous VL and SPVL. We then further demonstrate *in vitro* that E2 at physiological levels is able to restrict replication of a panel of HIV-1 T/F viruses in a dose-dependent manner. While we did not extensively study the function of testosterone, it

is interesting that we see the opposite pattern in men, wherein concentrations of testosterone, which is known to be highly anti-inflammatory, in the plasma at 1 month post-infection significantly correlated with SPVL (22).

Previous studies have observed varying effects of sex hormones on HIV-1 replication. Some of these demonstrated restriction of HIV-1 replication in monocyte derived macrophages (MDM), suggesting that our observations are not $CD4^+$ T cell specific (23-26). Several of these reports describe inhibition of HIV-1 replication by E2, but some report no effect or even enhancement of replication. However, direct comparison of these studies is complicated by differences in concentration of estrogen, cell type and donor sex, HIV-1 strain, and inclusion of additional sex steroid hormones in the experimental design. While some studies attempt to replicate physiological conditions by including both progesterone and estrogen, this complicates the understanding of the function of each individually (24). Importantly, E2 and progesterone are known to have many functions that directly oppose one another (27-29).

The studies described here were performed using a panel HIV-1 transmitted/founder (T/F) viruses directly cloned from patients infected with a single subtype C virus (30). Furthermore, we demonstrate consistent downregulation of replication in primary PBMC from a non-pregnant female donor of reproductive age with physiologically relevant concentrations of E2. We therefore believe that these experiments provide strong consistent evidence that physiologic concentrations of E2 in healthy women function to inhibit replication of HIV-1.

However, the mechanism behind a potential inhibition of HIV-1 replication by E2 are still a matter of debate. Tasker, *et al.* report that while E2 protects primary macrophages against HIV-1 infection via an entry-related mechanism, E2 did not affect surface expression of CD4 and HIV-1 co-receptors, nor HIV-1 attachment. Rather, they credit the induction of IFNα with

restricting viral replication. While they observe that E2 upregulated expression of some ISGs in MDMs from multiple donors, induction of host restriction factors APOBEC3G, APOBEC3F, or SAMHD1 was not consistent, with exception of APOBEC3A (26). While characterization of the effect of E2 on expression of antiviral genes is ongoing, our observations that a number of IFN-stimulated antiviral genes are more highly expressed in acutely HIV-1 infected women than men are consistent with these observations. Interestingly, we have identified SAMHD1 as being significantly more highly expressed by the $CD4^+$ T cells from acutely HIV-1 infected women than men. SAMHD1 and other type I IFN responsive genes including IRF7, DDX58, OAS2, and TRIM14, were both more highly expressed in $CD4^+$ T cells from women and negatively associated with VL, indicating that they may play a role in the comparative control of viral replication observed in women.

There is also recent evidence that estrogen and estrogen receptor-α (ERα) restrict HIV-1 reactivation from latency (31). Although the experimental questions differ, we believe our findings that implicated E2 in control of viral replication are consistent with these observations. It is likely that the same mechanisms responsible for ERα control of reactivation from latency simultaneously enable E2 to control HIV-1 replication. While Das *et al.* report recruitment of repressor complexes to the HIV-1 LTR by ERα, there are no obvious ER binding sites in the HIV-1 LTR, which suggests that the recruitment may be indirect (32, 33). A potential mediator of ERα recruitment is Sp1, which binds constitutively to three sites in the HIV-1 core promoter (32). Interactions between Sp1 and ERα and ERβ have been reported to recruit corepressor complexes at a number of genetic loci (34, 35). Relatedly, Szotek, *et al*. describe a mechanism by which E2 inhibits HIV-1 replication by inducing a complex formation between β-Catenin (a

regulator of Sp1) and ERα on the HIV-1 promoter to suppress transcription (36). Additional work will be necessary to address direct transcriptional control of HIV-1 by ERα.

In addition to exploring the impact of sex on VL and $CD4^+$ T cell phenotypes, we have also identified of a large number of genes whose expression in $CD4^+$ T cells was directly correlated with VL regardless of patient sex. Many of these are validated by previous work. For example, expression of ISGs are known to increase in HIV-1 infected individuals in various cell types (37-40). We found that some ISGs, including IRF1, IRF8 were positively associated with VL. However, we also identified a number of ISGs whose expression negatively correlates with VL, including IRF7 and IRF3. Type I IFNs engage in a number of antiviral activities, however they simultaneously contribute to the induction of chronic immune activation (41). This is evident in the rhesus macaque model, where IFNα administration was shown to initially upregulate expression of IFN-stimulated genes (ISGs) and prevent systemic SIV infection but continued IFNα treatment also resulted in accelerated $CD4^+$ T cell depletion and increased viremia (42). It is possible that the ISGs negatively correlated with VL are those that impart sufficient restriction on viral replication, although additional experimentation *in vitro* will be required to discern the causality of these relationships.

Finally, we identified a number of genes whose expression was significantly correlated with VL that have no known relationship to HIV-1 pathogenesis. For example, we identified a number of TRIM E3 ubiquitin ligases (TRIM2, TRIM27, TRIM46, TRIM58, TRIM62) whose expression negatively correlated with VL that have not been previously implicated in restriction HIV-1 replication. Further analyses and experimentation are likely to reveal additional potential regulators of HIV-1 pathogenesis.

Future Directions

We have identified here significant sex differences in HIV-1 pathogenesis in a cohort of ART-naïve individuals. However, it is critical to consider how the modern treatment setting affects the findings described herein. It is well established that relatively high levels of inflammation persist during ART treatment (43, 44). Although very little is known about how sex affects HIV-1 related inflammation, morbidity, and mortality in the context of ART, one recent study demonstrated that after ART initiation, levels of certain pro-inflammatory markers remained elevated in women to a greater extent than in men (45).

ART has also significantly increased lifespan of HIV-infected persons. One significant effect of this is the need for additional research in HIV-infected geriatric populations. This is additionally relevant to this work as women reaching menopausal age and undergo drastic changes in hormone levels. Our studies in the ZHERP cohort almost exclusively include individuals under the age of 40, so few of the women included are likely to have reached menopausal age. While there are very few studies looking at post-menopausal women infected with HIV-1, one recent report suggested that geriatric women were less likely to remain suppressed on ART than men (46). This would be in contrast to data of untreated infection in younger women which finds that women in many populations are more likely to suppress virus spontaneously, or maintain relative control compared to men (1-5).

Interestingly, a recent pediatric study also recently found that girls were less likely to maintain viral suppression than boys during ART interruption (47). If these observations are correct, there are a number of potential explanations. Importantly, the mechanisms of viral suppression on ART may be sufficiently different from those required for relative control of viremia that we and others observe in untreated women. While this work has focused primarily

on functions of $CD4^+$ T cells, recent data suggests that $CD8^+$ T cells play a critical role in viral suppression on ART via both cytolytic and non-cytolytic mechanisms (48, 49). Very little is known about sex differences in either general or HIV-specific $CD8^+$ T cell responses and therefore Further investigation will be required to determine whether sex plays a role in viral suppression by $CD8^+$ cells (15). Hormone levels may also play a role in these observations, as both geriatric patients and children would have significant differences in hormone levels than the reproductive-age women studied here. *In utero,* fetuses of both sexes are exposed to high levels of maternal and placental estrogens and progesterone. However, testosterone is produced by males from 8-14 weeks of gestation (50). Therefore, it is reasonable to hypothesize that differential immune regulation by sex hormones play a role in these paradoxical observations.

Adland, *et al.* additionally report a smaller proviral DNA burden in male children infected with HIV-1 *in utero* (47). In contrast, Das *et al.* observe a smaller inducible reservoir in adult women (33). These observations are not necessarily in conflict as there have often been significant differences observed between adult and pediatric HIV-1 infection (51, 52). Adland, *et al.* propose a mechanism by which female fetuses are preferentially infected due to increased activation of $CD4^+$ T cells, as we have observed in adult women. They further argue that the viruses transmitted to male fetuses are on average more IFN-sensitive and therefore more subject to immune control in the context of ART interruption (47). It is clear that studies in additional individuals in different age groups will be required to definitively determine on the impact of sex on the HIV-1 viral reservoir and whether it influences potential HIV-1 cure strategies in the future.

The mechanisms underlying sex-specific differences in HIV-1 pathogenesis are complex and multifaceted. We believe that this work sheds light on the significant immunological

differences that exist between men and women and elucidates how differences sex steroid hormones and type I IFN responses influence HIV-1 pathogenesis. It additionally provides strong justification for continued research into how sex differences in the immune response affect vaccination and cure strategies for HIV-1 and other viral pathogens.

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